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*SPECTROPHOTOMETRIC EXAMINATION OF HYDROGENASE AND NITROGENASE IN SOYBEAN NODULES AND AZOTOBACTER**

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Communicated February 21, 1957

In 1937 Wilson and Umbreit[1] discovered that molecular hydrogen specifically inhibits nitrogen fixation by the red clover plant in association with its symbiont, *Rhizobium trifolii*. They suggested that the inhibition might be associated with the presence of the enzyme hydrogenase in the nodule of the host plant, but efforts to demonstrate the enzyme have been generally negative. Tests for the enzyme have included a variety of possible acceptors and of enzyme preparations consisting of whole nodules, nodular extracts, the rhizobia separated from the nodule, as well as rhizobia grown on artificial media. Nevertheless, the hypothesis that hydrogenase is concerned with nitrogen fixation has been useful particularly with the asymbiotic agents, having led to the demonstration that several previously unsuspected genera can use N_2. It has also been helpful in studies of the mechanism of nitrogen fixation.[2, 3]

Another protein the exact role of which in nitrogen fixation is not established is the hemoglobin found in the nodule. Although most workers agree that probably it is directly concerned with nitrogen fixation, evidence in support of the view has not been extensive, and alternative functions exist.[4–6] Using the anaerobic nitrogen-fixing bacterium *Clostridium pasteurianum* and a spectrophotometric method, Shug, Hamilton, and Wilson[3] obtained results that provided important information with respect to the enzyme system concerned with nitrogen fixation in this organism. It was believed that application of this technique to the symbiotic system might aid in deciding the role, if any, for hydrogenase and hemoglobin in fixation by this system. At the same time, the aerobic nitrogen-fixing system in *Azotobacter vinelandii*, an organism that is believed not to contain hemoglobin, was also examined. This paper describes the results of the investigations.

MATERIALS AND METHODS

Field-grown soybeans of the Lincoln variety were harvested at 10 weeks. The nodules were stripped from the plants, freed of excess soil, and sorted, only the nodules of 4–6-mm. diameter being retained. These were placed in large Thunberg tubes under an atmosphere of hydrogen and were stored at −20° C. The whole procedure from harvest to storage required about one hour.

When desired, about 2 gm. of nodules were withdrawn from the Thunberg tubes and washed free of any remaining soil with distilled water saturated with H_2. The washed nodules were placed in 20 ml. of 0.05 *M* phosphate buffer, pH 7.0, and saturated with H_2, then crushed with a glass rod while a stream of H_2 was passed over the surface. This mixture was placed in the sonic field of a Raytheon 10-kc. magnetostrictive oscillator for 3 minutes, then centrifuged at 7,000 × *g* for 15 minutes. The supernatant was used directly or could be stored under H_2 for 24 hours without marked loss of activity. The resultant red extract usually had an optical density of 3–4 units at 400 mμ (i.e., less than 1 per cent incident light transmitted). All the foregoing steps were carried out between 0° and 5° C. under essentially anaerobic conditions, to prevent the formation of hemiglobin by the action of polyphenol oxidase.[7]

Commercial gases were used, certified to be of the following percentage purities: helium, 99.7; hydrogen, 99.4; nitrogen, 99.99. These were freed of oxygen by scrubbing with a solution of chromous chloride; the efficacy of this treatment was determined by examining the treated gas in a Consolidated-Nier isotope-ratio mass spectrometer. Vessels used were evacuated and filled by means of a vacuum line equipped with a mercury manometer and connected to the gas-line supply through a scrubbing tower. This line was considered to be freed of contamination when the gases originally present had been reduced to 0.001 per cent of their initial values.

The spectra were obtained with a Cary Model 11 automatic recording spectrophotometer equipped with ground-glass plates to eliminate the light-scattering properties of the turbid preparations. Time-course studies on the reduction of dyes were made on the Beckman DU spectrophotometer or in a conventional Warburg apparatus. All spectrophotometric measurements were made in 3-ml. Pyrex cuvettes of 1-cm. light path fitted with a Thunberg cap.

Azotobacter vinelandii strain O was grown in Burk's N-free medium (six liters) plus 1 mg. Fe/liter and 0.1 mg. Mo/liter in 9-liter serum bottles, through which air was sparged at 18 liters/min. The cells were harvested in a refrigerated Sharples centrifuge and stored at −20° C. Cell-free extracts were prepared by suspending 5-gm. cells in 30 ml. of 0.1 *M* phosphate buffer (pH 6.8) and breaking in the sonic field of a 10-kc. Raytheon magnetostrictive oscillator at 0°–5° C. for 10 minutes or in the 9-kc. instrument for 30 minutes. The unbroken cells and heavier debris were removed by centrifuging at 7,000 × *g* for 30 minutes at 0° C. The supernate was stored in large Thunberg tubes under H_2 at 2° C. The responses of this extract to the gases were measured in 3-ml. silica cells of 1-cm. light path, using the rapid recording split-beam spectrophotometer of Chance.[8] With this instrument the gases were bubbled through the extract during scanning.

RESULTS

Symbiotic System.—For purposes of orientation, the absorption spectra of various forms of the hemoglobin in the nodular extract were determined. A small portion of the extract was centrifuged at 144,000 × *g* for 1 hour; the supernatant, which contained the hemoglobin and had low light-scattering properties, was suitable for measuring absorption spectra. The reduced form was obtained by adding dithionite; the oxygenated, by shaking the reduced form in air; and the oxidized, by shaking an untreated preparation in air, allowing the nodular polyphenol oxidase to act. That

the oxidized form was methemoglobin (hemiglobin), and not oxidation products of phenolic substances, was demonstrated by making the fluoride and peroxide derivatives.

Figure 1 presents the absorption spectra of the various forms of the hemoglobin from soybean nodules. Figure 2 is an absorption spectrum of an extract kept under helium. This spectrum was made to determine the form of hemoglobin in these extracts. As may be seen by comparison with Figure 1, such an extract contains a mixture of hemoglobin and hemiglobin with the former predominating.

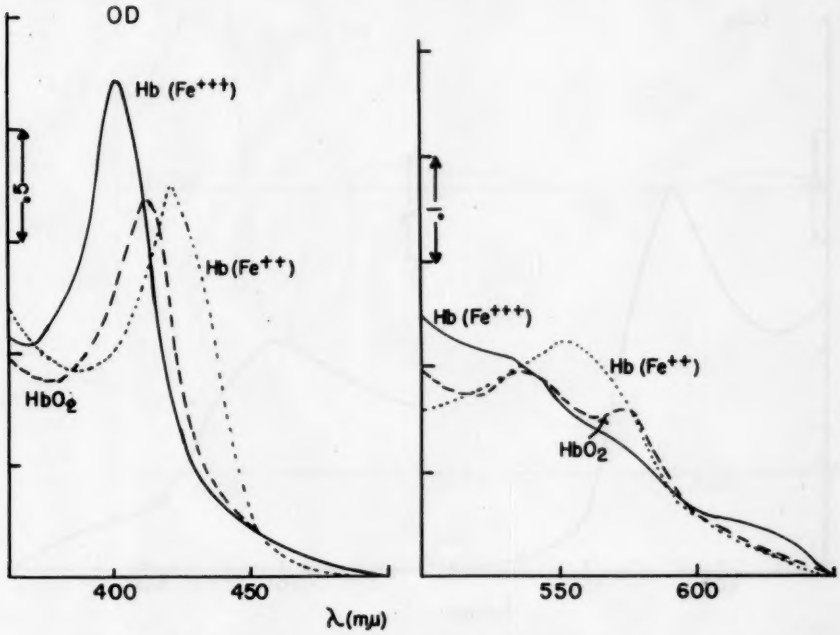

FIG. 1.—Spectra of different forms of soybean hemoglobin. Note that in Figures 1 and 2 the absolute absorption values are not plotted, as these vary with the preparation. The significant data are the wave lengths of the peaks, which serve to identify the form of the hemoglobin present.

The responses of the extract to H_2 and N_2 were determined by placing aliquots of the extract in each of three cuvettes, then filling the gas space with purified helium. Both absorption spectra and difference spectra were recorded in the Cary spectrophotometer. This step served to control the technique: the absorption spectra should be similar to that shown in Figure 2, and the difference spectra among the samples should be linear, with zero intercept and slope. After the control readings, the cuvettes were shaken and allowed to stand for a few minutes, and their spectra were again determined. This step served to check the purity of the gases and to test for leaks (any oxygen present will convert the hemoglobin to hemiglobin within a few minutes). If all these control steps were satisfactory, the gas phases were replaced with different gases—one with N_2, a second with H_2, and the third

with helium. Time was permitted for the gas and liquid phases to come to equilibrium; then the spectra were again recorded.

Figure 3 shows the difference spectra of the samples. The N_2 − He spectrum corresponds to a hem*i*globin-hem*o*globin difference spectrum; i.e., the direction of the changes indicates that the hem*o*globin was oxidized to hem*i*globin. The H_2 − He spectrum was the opposite—the hem*i*globin is reduced to hem*o*globin. Another experiment demonstrated that these changes did not occur when the hem*o*globin was combined with CO or when the preparation was aged.

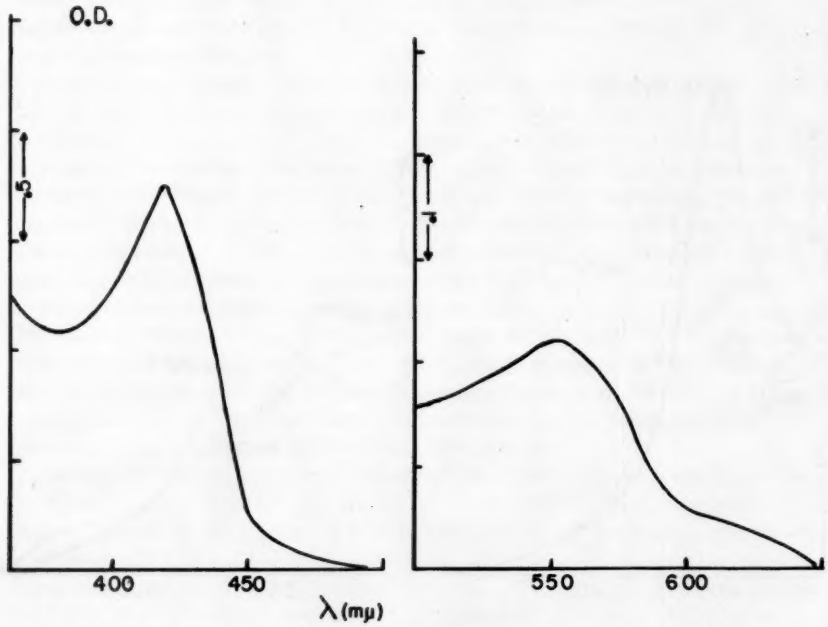

FIG. 2.—Spectrum of nodular extract from soybean in an atmosphere of helium.

In further investigations of the H_2 effect, the following possible acceptors were tested both manometrically and spectrophotometrically with and without added cofactors such as iron, molybdenum, flavins, nucleotides, and boiled extracts from various sources: methylene blue, viologens, ferricyanide, and nitrate. As in our earlier trials, the results were uniformly negative. Such negative results provide evidence that the spectral changes were not caused by contaminating bacteria—all other known hydrogenases will reduce one or more of these acceptors. However, as can be seen in Figure 4, the preparations will reduce added mammalian cytochrome c. Although the rate is rather slow, it is considerably higher than that observed with N_2 or He (endogenous reduction). One manometric experiment was made with cytochrome c as acceptor; the theoretical volume of H_2 disappeared if a correction for endogenous reduction was applied.

The presence of hydrogenase was further tested for by the deuterium-exchange

reaction. A slight but significant exchange was observed—only 0.011 atom per cent excess after 315 minutes. Recently, investigators in Professor R. H. Burris' laboratory in the Department of Biochemistry have obtained about a tenfold increase, but even this points to a very slow reaction.

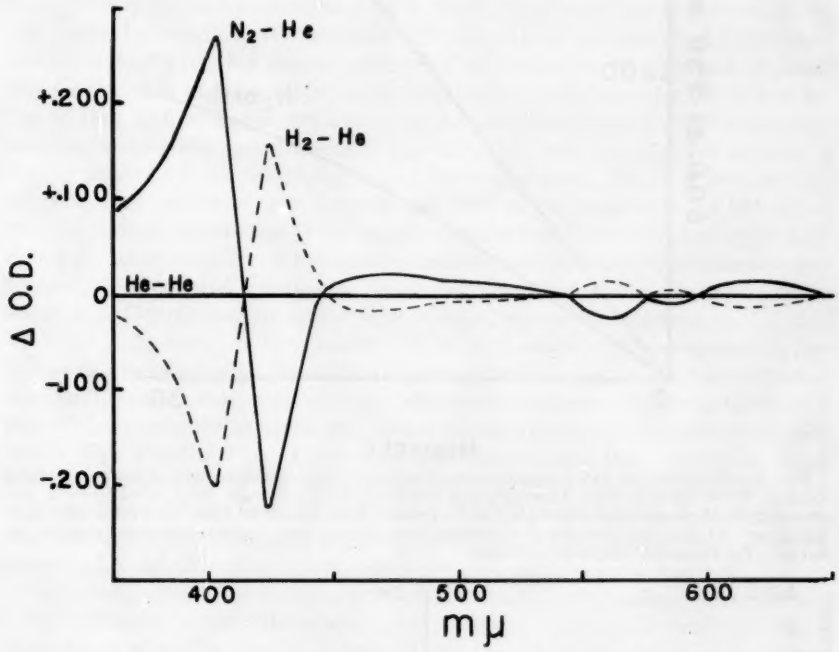

FIG. 3.—Difference spectra of a soybean nodular preparation exposed to H_2 and N_2. The baseline is a He — He difference spectrum.

Asymbiotic Azotobacter System.—Hydrogen gas was bubbled through two cuvettes containing the azotobacter extract until the last traces of O_2 were removed and the preparation was reduced. The difference spectrum between the two samples was recorded (base line in Fig. 5). Oxygen gas was then bubbled through one cuvette, and the difference spectrum between the oxidized and reduced forms was obtained. This spectrum in Figure 5 indicates reduction by H_2 of components with absorption peaks corresponding to cytochromes a_2, a_1, b, and c, as well as flavins. The role of diphosphopyridine nucleotide, if any, in the oxy-hydrogen reaction could not be determined, as the preparations were too turbid.

The effect of N_2 was obtained by reducing the preparation again with H_2, then bubbling N_2 through one cuvette. The $H_2 - N_2$ difference spectrum of Figure 5 indicates that N_2 oxidizes flavins and the "cytochrome b" component. Similar results have been obtained with both extracts and whole cells, using the Thunberg technique and reading the difference spectrum in the Cary Model 11 spectrophotometer.

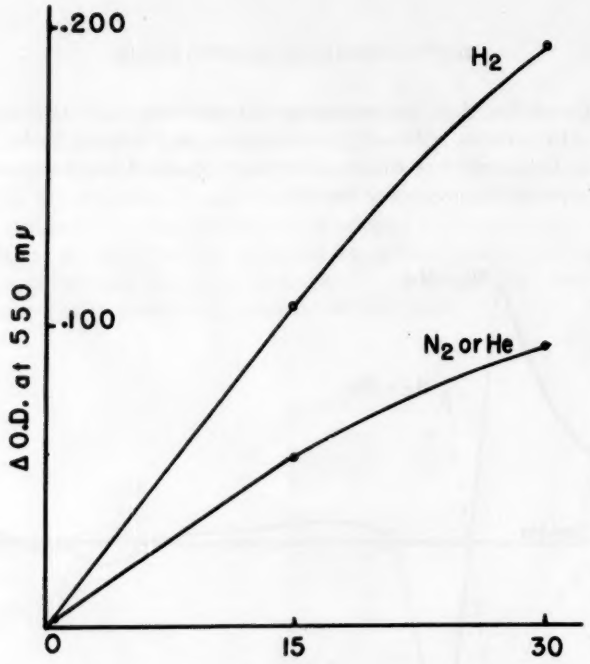

FIG. 4.—Reduction of added mammalian cytochrome c by H_2 in presence of soybean nodular extract. Pyrex cuvettes with Thunberg tops containing 0.4 ml. extract, 3 mg. cytochrome c, and enough 0.05 *M* phosphate buffer (pH 6.8) to make a final volume of 3 ml. were filled with purified gases. At zero time the extract was added from the side arm, and readings were taken at 550 mμ on a Beckman DU spectrophotometer.

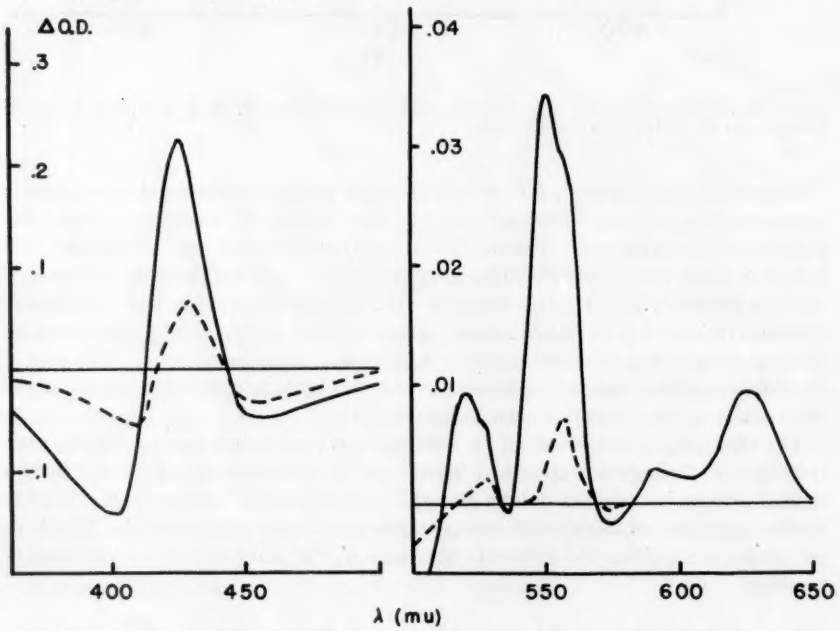

Fig. 5.—Difference spectra of *Azotobacter vinelandii* preparations treated with H_2, O_2, and N_2. The zero base line is a $H_2 - H_2$ spectrum, the dashed line is a $H_2 - N_2$ spectrum, and the solid line is a $H_2 - O_2$ spectrum.

DISCUSSION

Several reasons can be offered in support of the view that the N_2 effect reported here exists and that hemoglobin serves as an oxido-reduction catalyst in the nodule. The effect is specific: He, an inert gas, causes no changes, and H_2, a different one. The most obvious argument against this interpretation is that the oxidation of the hemoglobin is caused by contaminating O_2, but this gas could not be detected in the N_2 purified by standing over chromous chloride when it was examined in the mass spectrometer. Also, the oxygen-contamination argument requires one explanation for the effect of N_2, another for the action of H_2, and a third for the lack of action of He. Diffusing disks used to eliminate the light-scattering effect of the preparations occasionally cause apparent spectral shifts, but this possible artifact is argued against by the specificity of the shifts observed. Furthermore, the observations are consistent with known facts, such as the prevention by CO of the spectral shift at approximately the same concentration that inhibits nitrogen fixation in the intact nodule. If hemoglobin does play such a role in symbiotic nitrogen fixation, its subsequent reduction to repeat the cycle may be accomplished by flavoproteins, as Cormier and Rostorfer[9] have recently reported for bacteria.

Similar arguments for the presence of the long-sought-for hydrogenase in the nodule can be advanced. In addition to the observations reported here—spectral shift, exchange reaction, and reduction of added cytochrome c—Hoch, Little, and Burris[10] have recently observed that nodule extracts may under certain conditions evolve H_2. Confirmation of our previous observations that methylene blue, ferricyanide, nitrate, and the viologens are not reduced by H_2 does not appear to be such a serious objection as was once thought. Recent investigations have definitely established that a specificity for acceptors exists among hydrogenases from different sources, although all examined to date will use some of these acceptors.[11, 12]

The validity of the observation that N_2 oxidizes flavin and cytochrome components in the azotobacter rests on the same arguments already advanced for oxidation of the hemoglobin in the symbiotic system. Oxidation of cytochromes by acceptors other than O_2 is not unknown. Sato[13] reports that cytochrome components of a number of nitrate-reducing microörganisms are oxidized by nitrate, and Postgate[14] found that cytochrome c_3 is oxidized by sulfate in the sulfate-reducing bacterium *Desulfovibrio desulfuricans*.

SUMMARY

Sonic extracts of soybean nodules have been exposed to various gases and the spectral changes observed. In the presence of N_2 hemoglobin is oxidized to hemiglobin; the opposite reaction is observed with H_2. The extracts slowly reduce added mammalian cytochrome c and catalyze a slight deuterium-exchange reaction. In addition to their significance for the question of whether a hydrogenase exists in the nodule, these data support the suggestion that hemoglobin may act as an oxido-reduction catalyst in symbiotic nitrogen fixation.

The hydrogenase of *A. vinelandii* strain O will reduce compounds in this organism whose absorption peaks correspond to flavins and cytochromes a_2, a_1, b and c. N_2 appears specifically to oxidize flavins and the cytochrome b component.

* This research was supported in part by grants from the Atomic Energy Commission and the Research Committee from funds provided by the Wisconsin Alumni Research Foundation. The

authors thank Dr. Britton Chance for the hospitality of his laboratory, in which the spectra appearing in Fig. 5 were recorded.

[1] P. W. Wilson and W. W. Umbreit, *Arch. Mikrobiol.*, **8**, 440–457, 1937.

[2] R. H. Burris, in *Inorganic Nitrogen Metabolism* (Baltimore, Md.: Johns Hopkins Press, 1956), chap. iv, pp. 316–343.

[3] A. L. Shug, P. B. Hamilton, and P. W. Wilson, in *Inorganic Nitrogen Metabolism*, edited by W. D. McElroy and B. Glass (Baltimore, Md.: Johns Hopkins Press, 1956), chap. IV, pp. 344–360.

[4] A. I. Virtanen, J. Jorma, H. Linkola, and A. Lannasalmi, *Acta chem. Scand.*, **1**, 90–103, 1947.

[5] D. Keilin and J. D. Smith, *Nature*, **159**, 692–698, 1947.

[6] P. W. Wilson and R. H. Burris, *Biochem. J.*, **51**, 90–96, 1952.

[7] D. Keilin and Y. L. Wang, *Nature*, **155**, 227–232, 1945.

[8] B. Chance, *Nature*, **169**, 215–230, 1952.

[9] M. J. Cormier and H. H. Rostorfer, *Bacteriol. Proc.*, pp. 114–115, 1956.

[10] G. E. Hoch, H. N. Little, and R. H. Burris, *Nature*, **179**, 430–431, 1957.

[11] H. D. Peck, Jr., A. San Pietro, and H. Gest, these PROCEEDINGS, **42**, 13–19, 1956.

[12] A. I. Krasna and D. Rittenberg, these PROCEEDINGS, **42**, 180–185, 1956.

[13] R. Sato, in *Inorganic Nitrogen Metabolism*, edited by W. D. McElroy and B. Glass (Baltimore, Md.: Johns Hopkins Press, 1956), chap. ii, pp. 163–175.

[14] J. R. Postgate, *J. Gen. Microbiol.*, **14**, 545–572, 1956.

OPTICALLY ACTIVE COMPOUNDS FROM RACEMIC MIXTURES BY MEANS OF RANDOM DISTRIBUTION

BY JOHN H. NORTHROP

Communicated February 6, 1957

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Recent work indicates that many of the building stones of living organisms may have existed on the earth before the appearance of living systems.[1] This simplifies somewhat the problem of the origin of life; but, in order to fulfil the conditions necessary for the occurrence of a vital reaction, an optically active solution must be provided. Ordinary chemical reactions cannot meet the requirement, since, if optically active substances are formed at all, the two optically active isomers are produced in exactly equal proportions, and their solution will be optically inactive.

The classical methods of Pasteur for the isolation of an optically active compound from such a mixture will not suffice, since they depend directly or indirectly on living organisms. This fact has been cited as evidence that living organisms could not have been formed from inanimate matter.

Various suggestions have been made in order to avoid this difficulty.[2] Van't Hoff predicted that polarized light would cause asymmetric synthesis, and this prediction was confirmed. Cotton and others then suggested that the circularly polarized light on the earth might influence a reaction so as to favor the formation of one isomer. It was pointed out by Pearson that the formation or crystallization of the two isomers in exactly equal proportions is a statistical result, and hence, in rare cases, an excess of one or the other isomer might occur. Positive results by both methods have been reported.

A much simpler mechanism will serve to provide an optically active solution from

a racemic conglomerate (a mixture of individual *d* crystals and *l* crystals in equal proportions) such as may result from ordinary nonvital chemical reactions.

It is only necessary that the individual crystals be separated far enough from each other so that their solutions do not mix when the crystals dissolve. If the crystals are dissolved under these conditions, each optically active crystal will provide a small amount of an optically active solution.

The process might well occur in nature. If a solution of a racemic mixture formed as the result of an ordinary chemical reaction were to crystallize as a racemic conglomerate, and then evaporate to dryness, the crystals might easily be scattered by the wind or some other disturbance. This process would fulfil the required conditions, and each individual crystal, separated from the others, would supply an optically active solution. In this way an optically active solution would be made available for the first vital reaction.

[1] J. D. Bernal, *Physical Basis of Life* (London: Routledge & Kegan Paul, 1951); S. W. Fox, *Am. Scientist*, **44**, 347, 1956; A. I. Oparin, *Origin of Life* (New York: Macmillan Co., 1938).

[2] For a discussion of these problems see G. F. Gause, "Optical Activity and Living Matter," *Biodynamica* (Normandy, Mo.), 1941. E. Schrödinger, *What Is Life?* New York: (Macmillan Co., 1945); E. Schrödinger, in *History of Mathematics*, ed. Newman (New York: Simon & Schuster, 1956), **2**, 985; H. Weyl, in *History of Mathematics*, ed. Newman (New York: Simon & Schuster, 1956), **1**, 871.

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*THE GENETIC CONTROL OF ADENYLOSUCCINASE IN NEUROSPORA CRASSA**

BY NORMAN H. GILES, C. W. H. PARTRIDGE, AND NORMA J. NELSON

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Communicated by Karl Sax, February 14, 1957

In order to elucidate the mechanisms by which genes control enzyme formation, an experimental system favorable for both biochemical and genetical investigations is of paramount importance. Although an increasing number of instances are now known in which mutational changes have given rise to specific enzymatic deficiencies (Horowitz[1]), few of these systems have yet been characterized genetically in detail. The present paper will report initial results with a system in *Neurospora crassa* which appears to be particularly well suited for combined biochemical and genetical studies. A series of over twenty adenine-requiring mutants of independent origin has been obtained in one wild-type strain as a result of mutation at a single locus which is quite favorable for genetical studies, having suitable closely linked markers on either side. These mutants are all characterized by being deficient for a single enzyme involved in the terminal step in adenine biosynthesis—adenylosuccinase[2]—for which a convenient assay procedure is available. Furthermore, certain of the mutants are capable of reversion, and the characteristics of the resulting adenine-independent types can be investigated both biochemically and genetically.

Additionally, since all mutants were produced in a single wild-type strain, it

has been possible to test for heterocaryon formation and growth in the absence of adenine with the various strains. The unexpected result has been that certain combinations of mutants are able to form adenine-independent heterocaryons (bicaryons)† in which appreciable adenylosuccinase activity can be demonstrated, even though both components lack detectable adenylosuccinase activity. Thus the initial studies with this system are already proving to be of exceptional interest in relation to the problem of gene action in enzyme formation. Brief reports of certain of these results have been presented previously (Giles, Partridge, and Nelson[3]; Giles and Partridge[4]).

Production and Preliminary Classification of Mutants.—The adenine-requiring mutants used in these studies were obtained in wild-type strain 74A (of St. Lawrence) by application of the filtration-concentration technique of Woodward, deZeeuw, and Srb.[5] Untreated (control) macroconidia and macroconidia exposed to either X-rays or ultraviolet were utilized to obtain mutants. Following filtration, platings were made on minimal agar supplemented with adenine (and other supplements in certain experiments in which additional types of mutants were being sought), and single colonies were isolated for testing. The resulting adenine-requiring mutants (606 in number) were next screened for their response to hypoxanthine. The present studies are concerned with the resulting 47 adenine-specific mutants which do not grow on hypoxanthine.

Further characterization of these mutants was sought by means of heterocaryon tests for complementation resulting in growth in the absence of adenine. Utilizing a single, arbitrarily chosen strain as a standard, all other adenine-specific mutants were tested for heterocaryon complementation with this strain by mixing conidia of the two mutants on a minimal agar plate. On the basis of these tests, two distinct groups of mutants were detected: group E (27 mutants), which gave abundant growth with the tester within 3 days or less after inoculation in the absence of adenine, and group F (20 mutants) which gave no growth with the tester (Table 1).

Additional evidence for two distinct groups of mutants has been obtained from genetic studies. In crosses to wild type, individual mutants from the two groups

TABLE 1

ORIGIN OF ADENINE-SPECIFIC MUTANTS

GROUP	MUTANTS DERIVED FROM CONIDIA EXPOSED TO: X-rays	Ultraviolet	No Treatment	TOTALS
E	25	0	2 (E11 and E12)	27
F	17	1 (F19)	2 (F6 and F10)	20*

* Does not include mutant 44206, of ultraviolet origin (Barratt *et al.*, *Advances in Genetics*, **6**, 1, 1954).

exhibit generally regular 1:1 segregation in asci. Crosses between mutants in the two groups give approximately 25 per cent wild-type segregants, demonstrating that the two types are not linked. Further studies with the F mutants have shown that these constitute a group of allelic mutants in linkage group III located between *prol*-1 and *leu*-1 (Barratt *et al.*[6]). Heterocaryon and crossing tests have also shown that the F mutants are allelic with the previously known *ad-4* mutant (44206) reported as adenine-specific (and temperature-sensitive) by Mitchell and Houlahan.[7] Group E mutants constitute a new group of allelic, adenine-specific mutants not as yet located in any particular linkage group.

Biochemical Characterization of Mutants.—Biochemical studies of the two groups of mutants were initiated in an effort to determine what step in adenine synthesis is blocked in each type. Culture filtrates of E mutants were found to be active in supporting growth of all other groups of adenine mutants except F, a result which appeared to place E prior to F in the biosynthetic sequence. Ultraviolet absorption spectra of the culture filtrates of both types were obtained, and characteristic accumulation products were then separated by ion-exchange chromatography. The results of these studies, especially with F filtrates, have been described in a preliminary note (Partridge and Giles[8]) and are similar to those obtained independently for the F mutant 44206 by Whitfeld.[9] The evidence indicates that F mutants are blocked in the terminal step in adenine synthesis, involving the splitting of adenosine monophosphate succinate (AMP-S) to adenosine monophosphate (AMP).

Comparative studies of E and F mutants, including examination of the absorption spectra of filtrates from the single and double mutants, as well as identification of the accumulation product (hypoxanthine) in an E mutant responsible for the biological activity of its filtrate, led to the tentative conclusion that E mutants were blocked in the step prior to F in biosynthesis, namely, the conversion of inosine monophosphate (IMP) to adenosine monophosphate succinate (AMP-S). More recent observations (Partridge and Giles, unpublished) indicate that a revision of this view may be required. It now appears that F mutants are blocked in not one, but two, reactions, the second involving the splitting of 5-amino-4-imidazole-(N-succinylo-carboxamide) ribotide (SAICAR) to 5-amino-4-imidazole carboxamide ribotide (AICAR) (Buchanan[10]). The accumulation of hypoxanthine by E-F double mutants appears to place the E block prior to the two F blocks and hence to require a modification of the previous view[3, 4] regarding the step blocked in E mutants. Additionally, preliminary investigations of a third group of mutants (J) indicate that these may be considered basically adenine-specific, although they grow quite well on hypoxanthine when histidine is also present. The absorption spectrum of J-mutant filtrates is much like that of F mutants and suggests a close relationship between the substrates of the reactions under their respective control. Further studies of these relationships are under way. A study of the properties of double mutants involving J, E, and F types should help to decide the question of the relative positions of the compounds whose metabolism they affect, in the path of adenylic acid synthesis or as by-products thereof. All present evidence, however, supports the previous conclusion that F mutants are indeed blocked in the splitting of AMP-S to AMP.

The present experiments have been concerned principally with an analysis of the genetic control of this reaction, based on studies of the AMP-S splitting enzyme, adenylosuccinase, in wild type, F mutants, and revertants induced in F mutants.

Adenylosuccinase in Wild Type, Mutants, and Revertants.—On the basis of the evidence just discussed, indicating that F mutants accumulate AMP-S, it appeared that they might be defective with respect to the AMP-S splitting enzyme, adenylosuccinase (Carter and Cohen[11]). Hence tests for this enzyme were performed on mycelial extracts of wild type, F mutants, and certain other adenine mutants.

The following assay procedure was utilized: Mutant strains were grown with adenine sufficient for submaximal growth; prototrophic cultures were grown rou-

tinely on Fries minimal medium; mycelial pads were harvested by filtration, washed with water at 5° C., dried in the frozen state, weighed, and aliquot portions taken. The aliquots were crushed to powder, extracted by shaking in 0.05 *M* phosphate buffer, pH 7.0, for one hour at 5°, and centrifuged with refrigeration to remove visible sediment. Supernates were separated from lipid layers and sediment and were assayed either immediately or after one or two days' storage in a deep-freeze. Activity was not removed by filtration through a membrane of pore diameter $>1\ \mu$.

For assay, a fraction of 1 ml. of extract was brought to 3.3 ml. with the extraction buffer, containing AMP-S at a final concentration of 30μg/ml, in a 10-mm. silica cell. The absorption of the substrate-product mixture was followed continuously or at fixed intervals by comparison with an identical mixture, lacking substrate, in a matched reference cell. Activity was recorded as rate of change of absorbance at 280 mμ during the period of constant reaction rate, on a mycelial dry-weight basis.

A study of variation of activity in wild-type and E-mutant extracts with culture times from 32 to 60 hours at 35° indicated a relatively constant activity for both strains, with a maximum between 36 and 44 hours (approximately 30 per cent greater than earlier or later samples). Times of harvesting in subsequent experiments were chosen to correspond to a stage of development in the range of optimal activity. Similar activities were found in 25° and 35° cultures at equivalent stages of development, except in temperature-sensitive strains. High levels of activity were found in wild type (equal in both mating types) and in mutant strains of groups E, J, and B. However, the B strain, when grown on hypoxanthine in place of adenine, developed an extractable purple pigment in the mycelium and was found to be inactive. When the pigmented extract was mixed with the colorless adenine-grown extract of the same strain, it removed the activity of the latter to a large extent. No activity (not more than 0.1 per cent that of wild type) could be detected in most of the nineteen F strains tested, even under conditions of double the usual extract concentration and a fivefold increase in substrate concentration with prolonged incubation. The exceptions were a strain (F16) which showed a trace of growth on minimal medium and a barely detectable activity and strain 44206, which is prototrophic and enzymically active at temperatures up to about 32°. No significant inhibition of wild-type activity was noted upon addition of crude extracts of any of the F strains. Therefore, the enzyme is presumed to be absent or grossly altered in F. Tests with extracts of both wild type and 44206 showed that activity was proportional to extract concentration over the experimental range, giving no evidence of intrastrain inhibition in crude extracts. It is not impossible that fractionation or special treatments of the mutant extracts, or growth of mutants under different conditions, will reveal latent activity (Wagner[12]).

On the basis of the results just discussed, it can be concluded that forward mutation at the *ad-4* locus in the wild type to give adenine-requiring mutants results in the loss of activity of the enzyme adenylosuccinase. It thus became of interest to determine whether reversions in such mutants to adenine independence would result in the restoration of adenylosuccinase activity. For these studies a series of 15 revertants was produced by ultraviolet irradiation of F12 macroconidia. The

revertants were backcrossed to an F12 strain of opposite sex, and homocaryotic adenine-independent isolates were obtained for genetical and biochemical analysis. On the basis of backcrosses to wild type, all 15 revertants test as reverse mutations rather than as suppressor mutations.

Tests were next performed for adenylosuccinase activity, and these demonstrated that activity is present in all 15 revertants. Preliminary quantitative comparisons indicate, however, that the level of enzyme activity is markedly less (averaging about 50 per cent) in the revertant extracts than in those of wild type (Fig. 1). Further tests will be necessary to establish the reproducibility of these

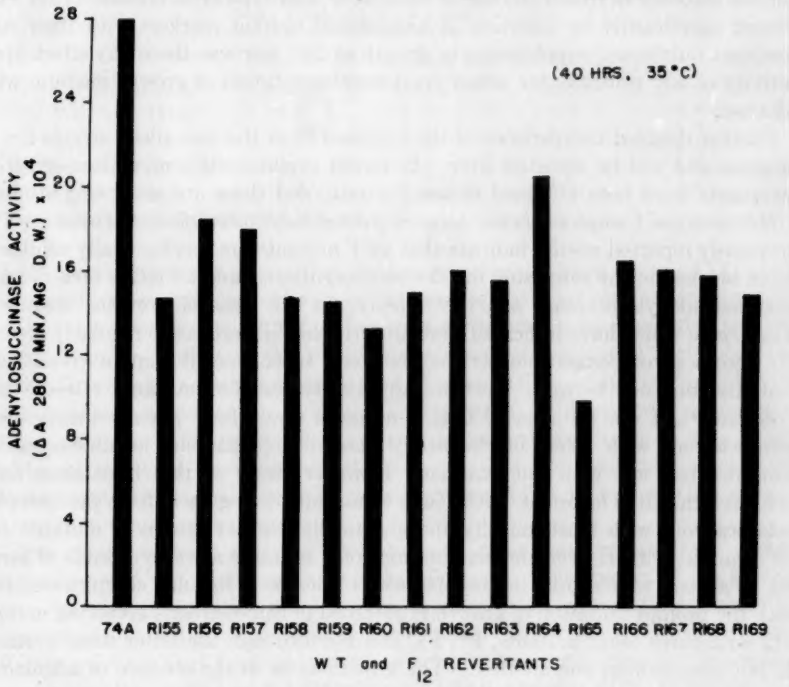

FIG. 1.—Comparative adenylosuccinase activity in wild type (74A) and 15 ultraviolet-induced revertants in the adenylosuccinase-deficient mutant F12.

quantitative comparisons, and to determine whether the observed quantitative differences among revertants arise exclusively from differences in the kinds of reverse mutational changes occurring at the F locus. A brief report of these studies has already been made (Giles and Partridge[4]), and additional detailed observations with these and other revertants will be published subsequently. Certain very recent observations indicate that the lower enzyme activities in at least some of the revertants may result from a more rapid loss of activity after extraction as compared with wild type.

Preliminary studies have also been made of adenylosuccinase in the temperature-sensitive mutant 44206 which grows in the absence of adenine at 25° but has an

absolute requirement at 35°. Mycelial extracts of this strain grown at 25° have about 25 per cent of wild-type activity, whereas those grown at 35° have substantially no activity. When the strain is grown at 32°, the upper limit of growth in the absence of adenine, extract activity is about 2 per cent that of wild type. An examination has been made of the relative thermostabilities of adenylosuccinase activity in extracts obtained from the temperature-sensitive mutant and from wild type. On the basis of comparative residual activities following incubation at 35° and at 48°, adenylosuccinase activity is much less stable in extracts from the temperature-sensitive mutant than in those from wild type. Neither the activities nor the stability in frozen storage of extracts of wild type or of mutant 44206 were altered significantly by insertion of biochemical mutant markers with their concomitant nutritional supplements in growth at 25°, nor was there any effect upon activity of any prototrophic strain upon supplementation of growth medium with adenine.

Further detailed comparisons of the enzymes from the two allelic strains are in progress and will be reported later. In recent experiments temperature-sensitive revertants have been obtained in one F strain, and these are also being studied.

Heterocaryon Complementation between Adenylosuccinase-deficient Mutants.—The previously reported results indicate that all F mutants are biochemically similar in being blocked in the same step in adenine biosynthesis, and all either lack or have modified adenylosuccinase activity. Hence, on the basis of previous studies in *Neurospora* which have indicated that prototrophic bicaryons are regularly formed (in heterocaryon-compatible strains) between biochemically unlike (nonallelic) mutants, but not between biochemically similar (allelic) mutants (Beadle and Coonradt[13]), it was anticipated that F mutants would form adenine-independent heterocaryons with other biochemically dissimilar (nonallelic) adenine-requiring mutants, but not with one another. However, tests of this hypothesis have indicated that it is incorrect. Although F mutants do regularly form prototrophic heterocaryons with biochemically and genetically distinct groups of mutants (as, for example, with E), certain combinations of F mutants are also capable of forming bicaryons which grow in the absence of adenine. Regular complementation with the prompt initiation of growth is obtained in combinations involving mutant F12 with three other mutants, F1, F4, and F5, although the latter three mutants do not complement one another. The growth rates in the absence of adenine of such heterocaryons compared to those of wild type and of a heterocaryon between F12 and an E mutant (E4), as measured at two temperatures, are shown in Table 2. It will be noted that at both 25° and 35° all bicaryons grow at rates comparable to wild type and are not markedly stimulated by adenine, with one exception, F12 + F1, which grows much more slowly at 35° and responds to exogenous adenine. Instances of complementation involving F mutants other than F12 have also been obtained, but these are characterized by delayed initiation of growth and much slower rates on minimal. For the present, attention has been centered on the three heterocaryons cited. In certain of the numerous combinations of F mutants which do not complement, forced heterocaryons have been made, and in all instances these have been found to require exogenous adenine for growth. Attempts to modify these negative results by employing heterocaryons containing various nuclear ratios have been unsuccessful.

The occurrence of adenine-independent heterocaryons between adenine-requiring, adenylosuccinase-deficient mutants immediately raised the question whether enzyme activity is present in such heterocaryons. Tests of mycelial extracts have indeed shown that adenylosuccinase activity is present. As shown in Figure 2, heterocaryons between F12 and F4 and F12 and F5 have about one-quarter the activity of wild type and about one-half that of a heterocaryon between F12 and E4.

TABLE 2

GROWTH RATES OF HETEROCARYONS BETWEEN F MUTANTS COMPARED WITH THOSE OF WILD TYPE AND AN E + F HETEROCARYON*

MUTANT COMBINATION	GROWTH RATE (MM/HR)			
	25°		35°	
	Minimal	Adenine	Minimal	Adenine
Wild type (74A)	3.0	3.2	4.0	4.2
F12 + E4	3.2	3.5	4.5	4.9
F12 + F1	3.0	3.5	2.0	4.4
F12 + F4	3.1	3.6	3.9	4.5
F12 + F5	2.8	3.5	4.3	4.6

* Tubes in duplicate; adenine supplement, 50 μg/ml.

A later experiment has shown that the heterocaryon between F1 and F12, which is temperature-sensitive, provides an extract only about 6 per cent as active as that of wild type, when grown at 25° either with or without exogenous adenine. Its activity relative to that of the non-temperature-sensitive intra-F heterocaryons is then 25 per cent, a ratio similar to that of strain 44206 compared to wild type. The fact that a heterocaryon of F1 and F12 grows at wild-type rate on minimal medium, whereas it produces only 6 per cent of wild-type activity in its extract, might suggest that at least one step (the terminal) in the production of an essential cellular constituent (AMP) may be greatly oversupplied in terms of its specific enzyme in a normal organism.

These results indicate that the heterocaryons, even those within the F group, can synthesize AMP by the same route as wild type. The spectra of the heterocaryon filtrates are indistinguishable from those of wild type and thus provide no indication of an alternative pathway in these special cases.

Recent evidence that F mutants also lack activity for another reaction in adenine biosynthesis, the splitting of SAICAR to AICAR (Buchanan[10]; Partridge and Giles, unpublished), appeared to reopen the possibility that complementation might arise on the basis of these two reactions—one mutant (e.g., F12) lacking activity for one, the other three mutants lacking activity for the other. However, tests of mycelial extracts of the four mutants indicate that all lack enzyme activity for this second substrate, an activity which can be demonstrated in wild type, in F revertants, and also in heterocaryons of the complementary F mutants. No significant differences in relative activities for the two reactions have been found in any instance.

Genetic tests have been made to determine whether the adenine-independent cultures obtained from the various F-mutant combinations are indeed heterocaryons with mutant nuclei only and do not contain wild-type nuclei arising as a result of reversion or some other genetic change. In all instances tested, such

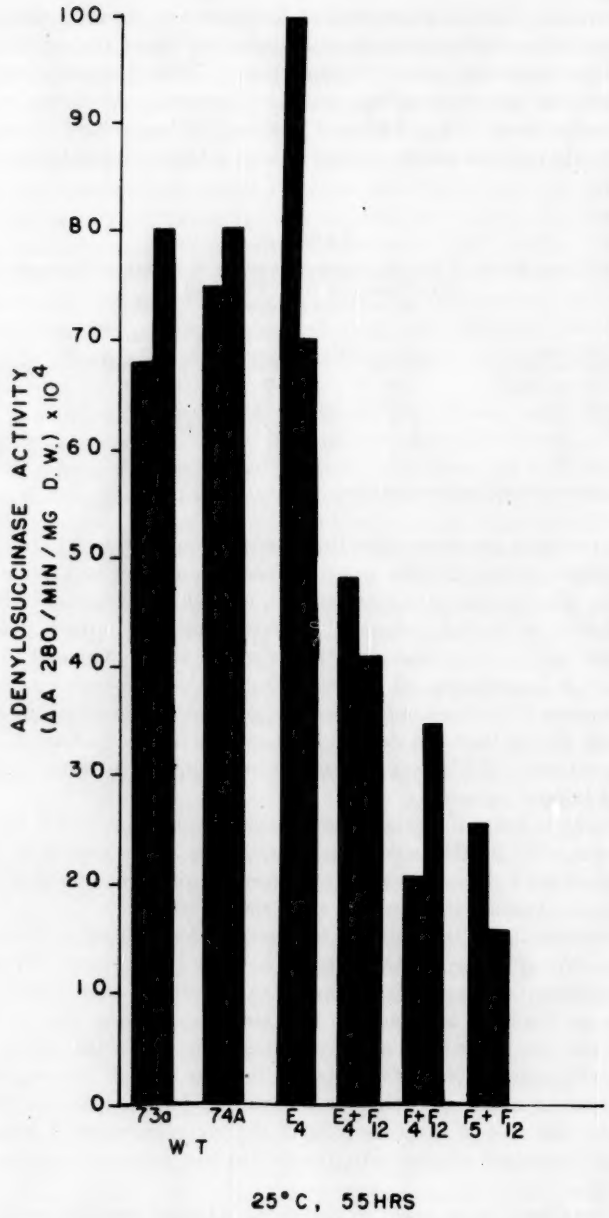

FIG. 2.—Comparative adenylosuccinase activities in mycelial extracts of wild types (74A and 73a), an E mutant (E4), and adenine-independent heterocaryons between F mutants (F4 + F12; F5 + F12). Results of duplicate tests are indicated.

presumptive heterocaryons can be resolved by hyphal-tip isolations and conidial platings to recover the two original mutant types, which can be identified by their pattern of heterocaryon formation with F testers. Despite extensive tests, no evidence has been obtained for the presence of other than mutant nuclei in these heterocaryons. In addition, preliminary tests using genetically marked strains have given no indication of mitotic recombination between the two types of mutant nuclei in such heterocaryons.

In view of the complementation in vivo between certain F mutants, resulting in the restoration of adenylosuccinase activity in heterocaryons, preliminary attempts have been made to detect such activity in an in vitro system in which mycelial extracts from two complementary mutant types were mixed. To date, such tests have given only negative results.

Genetic Relationships of Adenylosuccinase-deficient Mutants.—As already indicated, all F mutants are located in linkage group III between the same two markers. Intensive genetic analyses of these apparently allelic mutants have only recently been initiated, now that satisfactorily marked stocks are available. Analyses of crosses between various mutants are proving to be difficult because of sterility and poor ascospore viability, especially in serial isolations. However, it is already clear that crosses of certain mutants yield a low frequency of adenine-independent progeny, whereas selfings of the parents give only adenineless progeny.

To date, most adenine-independent segregants have proved to be pseudo-wild[14] rather than true wild types. Pseudo-wild types have resulted only from crosses of mutants which complement in heterocaryons. Only a few instances of true wild types have been detected as yet. In crosses where markers have been present, the origin of these adenine prototrophs has been associated with marker recombination. However, present crossing results are not extensive enough to determine what type of recombination (whether "orthodox" or involving "conversion") occurs between various mutants. It is expected that current studies will clarify further the genetic relationships of the various F mutants.

A particular study has been made of the inheritance of the pattern of heterocaryon complementation in F mutants exhibiting this behavior. This analysis involved crosses of such F mutants to wild type and tests of the heterocaryon responses of these F mutants in the first and subsequent generations. In all tests to date, F segregants which are heterocaryon-positive (capable of forming heterocaryons on minimal with compatible, biochemically unrelated strains) have shown the same pattern of heterocaryon response as the parental strain. Further tests have involved crosses of heterocaryon-positive strains of two F mutants (capable of complementation), one strain carrying a closely linked marker gene. Tests of segregants from such crosses indicate that a given pattern of heterocaryon complementation is linked with the marker, as is expected if this response is a regular characteristic of a particular adenine mutant.

In summary, in all tests to date, it has not been possible to separate the pattern of heterocaryon complementation from the particular adenine mutant with which it was originally associated. Hence it appears highly probable that this pattern is a result of the particular type of mutational event producing a given adenine mutant.

Discussion.—The preceding evidence indicates that one group of allelic, adenine-specific mutants in *N. crassa* (group F—or *ad-4*—containing 21 mutants of independent origin) can be characterized biochemically as being blocked in the terminal step in adenine biosynthesis, involving the splitting of AMP-S to AMP, as a result of the absence or impairment in these mutants of the AMP-S splitting enzyme, adenylosuccinase. This enzyme is present in wild type and in other biochemically distinct mutants. One of the *ad-4* mutants is temperature-sensitive and, when grown at 25° in the absence of adenine, contains appreciable adenylosuccinase activity. There is preliminary evidence that the enzyme from this mutant is much more thermolabile than that from wild type.

Furthermore, it has been demonstrated that certain of the F mutants lacking detectable adenylosuccinase activity can revert either spontaneously or following ultraviolet irradiation to give adenine-independent isolates testing as reverse mutations. Fifteen such revertants have been shown to possess adenylosuccinase activity, although their average activity is only about 50 per cent that of wild type, and none is equal to wild type in activity, at least after frozen storage of their extracts.

The foregoing results establish that in this instance forward mutation in the wild type to adenine requirement results in the loss of activity of a specific enzyme involved in adenine biosynthesis, and that reverse mutation results in the restoration of this activity. Additionally, it appears, on the basis of qualitative (temperature-sensitivity) and quantitative enzyme activity tests, that changes at the *ad-4* locus arising from mutation in either the forward or the reverse direction may produce diverse mutant and revertant types. Hence this particular situation provides an exceptionally favorable system for further combined biochemical and genetical studies of mutants arising by both forward and reverse mutational events at a single gene locus.

Of particular interest in the present investigation has been the unexpected finding that certain combinations of *ad-4* mutants are able to form heterocaryons (bicaryons) which can grow in the absence of adenine, and that adenylosuccinase activity, although absent from the mutants, is present in the heterocaryons. This type of complementation takes place between certain mutant combinations only. It is also clear that the absence of complementation does not result from a failure of heterocaryon formation, since heterocaryons have been forced with several such combinations, and these require adenine for growth. Several possible interpretations of these observations have been considered.

The occurrence between *ad-4* mutants of complementation essentially similar to that observed between biochemically unrelated types, in which different reactions are known to be blocked, raises the question as to whether the presumed single-step reaction from AMP-S to AMP may actually consist of two steps. However, all presently available evidence tends to refute this possibility. Biochemical studies with yeast and pigeon liver favor the view that a single-step reaction is involved, catalyzed by a single enzyme (Carter and Cohen[11]). In the present material no qualitative differences were detected among the various *ad-4* mutants, such as the accumulation of intermediates other than AMP-S. Furthermore, in vitro mixing of extracts of the F mutants capable of complementation

in heterocaryons failed to produce any activity for the over-all reaction. In fact, no spectral changes were observed in AMP-S in the presence of such extracts, either separate or mixed, whereas it might have been anticipated that, since the enzyme is soluble in active strains, an indication of one or both of the hypothetical component reactions would have been observed in the presence of separate extracts, and certainly an indication of the over-all reaction should have appeared in a mixture of such hypothetical soluble component enzymes.

The possibility that complementation might arise on the basis of differential activities for the splitting of SAICAR and AMP-S also appears to have been eliminated by the failure to demonstrate any activity for either substrate in any of the four F mutants concerned. At present it appears likely that a single enzyme, deficient in all the *ad-4* mutants, is able to catalyze both splitting reactions, which are chemically quite similar.

Additional general evidence against the view that two reactions may be involved comes from the fact that the *ad-4* mutants constitute not two classes, but three, with respect to their complementation. It is thus necessary to assume that the majority of the mutants lack both postulated activities. However, it is clear that the majority of these mutants must have arisen by single mutational events, which would require a remarkably close juxtaposition of the loci presumably controlling the distinct reactions. In the absence of any evidence to the contrary, it seems reasonable to assume that the splitting of AMP-S involves a single reaction catalyzed by a single enzyme. Furthermore, all available data support the view that mutation at only one locus results in the absence or modification of adenylosuccinase.

Another possibility considered was that heterocaryons able to grow in the absence of adenine could do so because they contained wild-type nuclei. However, the following evidence is against this view. (1) Such heterocaryons can be resolved by hyphal-tip isolations and conidial platings, with the recovery of adenine-requiring colonies containing either one or the other of the two original types of mutant nuclei as determined by their heterocaryon responses with the parental tester stocks. Adenine-independent colonies are also recovered from macroconidial platings, but all such colonies tested have proved to be heterocaryons, undoubtedly arising from conidia containing both types of mutant nuclei. Extensive tests have failed to produce any evidence that true wild-type nuclei are present in these heterocaryons. (2) Preliminary tests for mitotic recombination involving linkage group III markers have given negative results. (3) Quantitative comparisons of adenylosuccinase activity in E-F heterocaryons with that in intra-F heterocaryons would appear to require that about one-quarter of the nuclei in the latter heterocaryons be wild type in genotype, which is obviously not the case. Thus these results appear to eliminate the possibility that mitotic recombination or some type of nuclear interaction analogous to transformation or transduction is occurring to produce permanently wild-type nuclei in these adenine-independent heterocaryons.

The final hypothesis to be considered, and the one that seems most probable on the basis of present evidence, is that heterocaryon complementation arises from an interaction involving two different types of mutant nuclei present in a common cytoplasm, resulting in the synthesis of adenylosuccinase, and that this complementation occurs without any permanent genetic change having taken place in the

nuclei involved. As yet there is no evidence as to what the nature of such an interaction may be. It is possible that the presence of one type of nucleus may so modify cytoplasmic conditions that the other nucleus becomes capable of effecting adenylosuccinase synthesis. A more likely possibility would appear to be that some type of nuclear product interaction is involved. The present genetic evidence indicates that a given pattern of complementation is a genetic characteristic of a particular mutant and inseparable from the mutant locus. Thus the presence of a suppressor in one adenine strain, specific for another strain, and hence permitting growth in a heterocaryon, is ruled out (unless the suppressor locus is exceedingly closely linked with the *ad-4* locus). A reasonable possibility would seem to be that, in cases where complementation occurs, the two mutants involved have arisen as a result of mutational changes at different sites in the *ad-4* locus. Where complementation fails, the two mutants may have a certain portion of the locus damaged in common. Thus the mechanism could be visualized as involving cytoplasmic interaction between two types of imperfect templates resulting in the formation of perfect templates for enzyme formation, or a co-operative functioning of two imperfect templates to produce a normal enzyme. It will be of interest to determine whether this hypothesis can be correlated with the behavior of these mutants on crossing, especially with respect to the origin of wild types. It is evident, of course, that other interaction mechanisms can also be hypothesized.

The occurrence of prototrophic heterocaryons between *ad-4* mutants provides further evidence that the heterocaryon test is not a consistent one for detecting allelism (Mitchell[15]). Furthermore, although it has not been possible to obtain stable diploid heterozygotes in *Neurospora*, the evidence that adenine-independent pseudo-wild types arise from crosses of mutants which complement, strongly supports the view that the characteristics of such heterozygotes would be similar to those of heterocaryons with respect to enzyme formation. Thus the present results appear to make more difficult the general application of the *cis-trans* position effect test to delimit a locus as a functional unit (Benzer[16]), at least if functional unity is defined as control over the synthesis of a single enzyme.

Summary.—Combined genetic and biochemical studies have been performed on a series of adenine-specific mutants in *N. crassa*. Twenty-one mutants of independent origin have arisen as a result of mutation at a single locus (*ad-4*) in linkage group III between two closely linked marker genes. Initial crossing analyses with certain of these mutants indicate that adenine-independent isolates arise with a low frequency in some crosses, and that the majority of these types are pseudo-wilds, although true wild types occur in certain combinations. The recombination mechanism associated with the origin of wild types has not yet been established.

Biochemically, all the mutants have been found to be blocked in the terminal step in adenine biosynthesis, involving the splitting of adenosine monophosphate succinate (AMP-S) to adenosine monophosphate (AMP), and to lack or have impaired activity for the AMP-S splitting enzyme, adenylosuccinase. One of the mutants is temperature-sensitive and produces an adenylosuccinase which in crude extracts is much more thermolabile than that from wild type. Certain of the *ad-4* mutants are capable of reverse mutation to adenine-independent phenotypes, and these have been shown to possess restored adenylosuccinase activity, although at levels (or of stabilities) below that of wild type.

These results establish that in this instance forward mutation at a single locus in the wild type to adenine requirement results in the loss of activity of a specific enzyme involved in adenine biosynthesis and that reverse mutation to adenine independence results in the restoration of this activity. Additionally, changes at this locus, arising from either forward or reverse mutational events, may produce diverse mutant and revertant types as judged by qualitative and quantitative tests of enzyme activity.

Certain combinations of *ad-4* mutants have been found to form heterocaryons (bicaryons) able to grow in the absence of adenine. Although the individual mutants lack detectable adenylosuccinase activity, this enzyme is synthesized by the bicaryons. Possible mechanisms for this unexpected type of complementation between alleles in enzyme formation are discussed, along with its implications for biochemical genetic theory in general.

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† This term, rather than dicaryon which generally has a more restricted meaning, is used to designate a heterocaryon having only two kinds of mutant nuclei. Heterocaryons having more than two kinds of mutant nuclei, e.g., tricaryons, have not yet been investigated in these studies.

[1] N. H. Horowitz, *Federation Proc.*, **15**, 818, 1956.

[2] C. E. Carter, *Ann. Rev. Biochem.*, **25**, 123, 1956.

[3] N. H. Giles, C. W. H. Partridge, and N. J. Nelson, *Proc. Japan. Genetics Symposia*, 1956 (in press).

[4] N. H. Giles and C. W. H. Partridge, *Genetics*, **41**, 645, 1956 (abstr.).

[5] V. W. Woodward, J. R. deZeeuw, and A. M. Srb, these PROCEEDINGS, **40**, 192, 1954.

[6] R. W. Barratt, D. Newmeyer, D. D. Perkins, and L. Garnjobst, *Advances in Genetics*, **6**, 1, 1954.

[7] H. K. Mitchell and M. B. Houlahan, *Federation Proc.*, **5**, 370, 1946.

[8] C. W. H. Partridge and N. H. Giles, *Arch. Biochem. and Biophys.* (in press).

[9] P. Whitfeld, *Arch. Biochem. and Biophys.*, **65**, 585, 1956.

[10] J. M. Buchanan, personal communication.

[11] C. E. Carter and L. H. Cohen, *J. Biol. Chem.*, **222**, 17, 1956.

[12] R. P. Wagner and C. H. Haddox, *Am. Naturalist*, **85**, 319, 1951.

[13] G. W. Beadle and V. L. Coonradt, *Genetics*, **29**, 291, 1944.

[14] M. B. Mitchell, T. H. Pittenger, and H. K. Mitchell, these PROCEEDINGS, **38**, 589, 1952.

[15] M. B. Mitchell, *Compt. rend. trav. Lab. Carlsberg, Sér. physiol.*, **26**, 285, 1956.

[16] S. Benzer, these PROCEEDINGS, **41**, 344, 1955.

*ARE ALL PRODUCTS OF SPERMATOGENESIS REGULARLY FUNCTIONAL?**

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It is generally accepted that the four products of meiosis in the male, of *Drosophila* as well as of most other animals, are functional, and this view is not inconsistent with the bulk of histological and genetic evidence available. This is an account of a simple genetic experiment with *D. melanogaster*, the results of which cast some doubt on the universality of that generalization.

The experiments described here were conducted to obtain information about the relative frequencies of the different kinds of sperm produced by males carrying the translocation $T(1, 4)B^S$ [Translocation (1, 4) Bar of Stone[1]]. This is a translocation between X chromosome and the minute fourth chromosome. The approximate breaks in the normal chromosomes that gave rise to the translocation, and the resulting rearranged chromosomes, are shown in Figure 1.

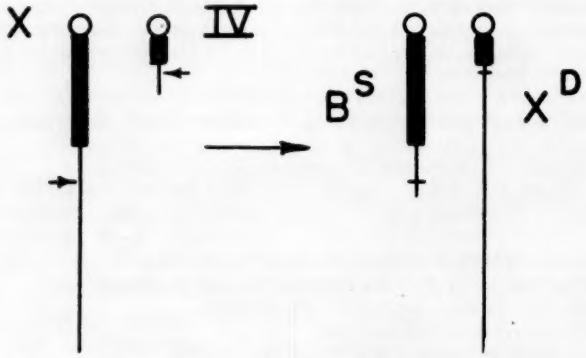

FIG. 1.—The approximate position of the breakpoints on the normal X and fourth chromosomes that have given rise, by reciprocal translocation, to $T(1, 4)\ B^S$.

Males carrying this translocation also carry a Y chromosome and a normal fourth chromosome. It is observed that such males produce sperm with either that part of the translocation with the base of the X chromosome, or the Y chromosome, but not both, and either that part of the translocation with the free end of the X, or the fourth chromosome, but not both. If we designate the segment of the translocation carrying the distal end of the X by A, the fourth chromosome by A', the segment of the translocation carrying the base of the X by B, and the Y chromosome by B', then A and A' behave like homologues, as do B and B'. Under these conditions, such a male produces four gamete types: AB, $A'B'$, AB', and $A'B$. Figure 2 shows the orientation of the paired homologues with respect to the plane of separation in the spermatocyte that would give rise to these combinations.

When males carrying the translocation are mated to attached-X females with a Y chromosome, AB, $A'B'$, and $A'B$ are recoverable and AB' is lethal. To recover AB', the Y chromosome of the female is replaced by the proximal segment of the translocation, B. The recoverable gamete types in this case are $A'B'$, AB', and

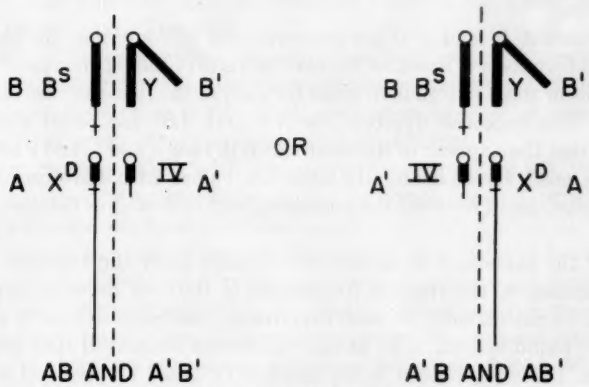

FIG. 2.—The two kinds of orientation of the paired homologues that give rise to the four gamete types.

$A'B$, with AB semilethal or lethal. The results from these two kinds of matings can be combined to determine the relative frequencies of recovery of the four gamete types.

The results of four experimental runs are given in Table 1. Experiments I and II differ in that the first involves mating the translocation male to an attached-

TABLE 1

NUMBERS OF PROGENY IN FOUR DIFFERENT RUNS FROM MATING TRANSLOCATION-BEARING MALES TO ATTACHED-X FEMALES

	Female Gamete Types	Male Gamete Types: AB ($X^D + B^S$)	$A'B'$ (IV + Y)	AB' (X^D + Y)	$A'B$ (IV + B^S)
Expt. I, *a* and *b*	XX	Lethal	+ *a* 591 (599) *b* 438 (411)	Lethal	*y* B^S *a* 1349 (1341) *b* 891 (918)
	Y	*y* B^S *a* 847 (839) *b* 566 (574)	Lethal	Lethal	Lethal
Expt. II, *c* and *d*	XX	Lethal	+ *c* 273 (287) *d* 275 (280)	Lethal	*y* B^S *c* 655 (641) *d* 632 (627)
	B^S	Semilethal	Lethal	B^S *c* 196 (175) *d* 150 (171)	Lethal
Relative frequencies		0.2651	0.1911	0.1163	0.4275

X female with a Y chromosome (unmarked); the second, to an attached-X female with section B of the translocation. In sets b and d the males carry a fourth chromosome marked with ci^D; in a and c the fourth is unmarked. In the male the component A is marked with yellow, B with Bar, and B' with the normal allele of

yellow in all cases. In the female the attached-X is homozygous for yellow, the Y is unmarked, and the component *B* (used in Experiment II) carries Bar. The phenotypes of each recoverable class are given in the appropriate square, and after the letter of each of the four runs is the number observed in that class, followed by the expected number in parentheses, if the same ratios of types are assumed to occur in all four runs.

Since gametes $A'B'$ and $A'B$ are recoverable in all four runs, the calculation of their expected numbers is based on the over-all ratio of these two types, 1,577:3,527, and the totals of these two gamete types for each of the four runs are subdivided in that ratio. The expected numbers for AB and AB' are based simply on the assumption that they appear in the same ratios in runs *a* and *b*, and *c* and *d*, respectively. The good agreement of the expected values with the observed suggests that the frequencies of recovery are consistent within as well as between the experiments.

Since half the gametes from attached-X females carry the attached X and half carry its homologue, the relative frequencies of the four different types of male gametes can be calculated. In each experiment, one gamete type is missing, but since two are found in both, a set of four values can be derived that best fit all the observations. These are given in the last line of Table 1.[2] Several puzzling features surround these derived gamete frequencies.

1. The values appear to bear no relation to each other and might be dismissed as "biological variability" were it not that they are reproducible.

2. Complementary classes are not equal. The most obvious inequality is that of 0.428 to 0.116, clearly a striking difference.

3. Following usual translocation terminology, the total of the two "adjacent" classes exceeds that of the "alternate" classes (54 versus 46 per cent).

These values were accepted as the best estimates of the frequencies of recovery of the four types of gametes from the male. The possibility of differential viability of the different classes on which the derived values depend, always a difficult problem to handle, was considered and dismissed as inadequate, but this point will be dealt with more effectively in the section on zygote mortality.

A relation between the frequencies of the sperm types is revealed by a simple but slightly unorthodox approach. Let us inquire as to the over-all frequency with which each of the four components is found among all the recovered gametes. These values are as follows: A, 0.381 (0.265 + 0.116); A', 0.619 (0.428 + 0.191); B, 0.693 (0.265 + 0.428); B', 0.307 (0.116 + 0.191). Now we can ask for the frequencies of expectation of the four combinations; these will simply be the appropriate cross-products of the values given, and they turn out to be 0.264, 0.190, 0.117, and 0.429 for AB, $A'B'$, AB', and $A'B$, respectively. Since the observed values, given in the same order, are 0.265, 0.191, 0.116, and 0.428, it can be seen that the agreement is extraordinarily good.

The significance of the algebraic operation is this: If we attach to each of the components a probability that it will be found in a fertilized egg, simply by multiplying probabilities we get the probability that two nonhomologous (independent) components will be found together. Although this is similar to a statement of a basic rule of probability, it is astonishing that the results from the experiments can be treated in this fashion with such close agreement between results and these de-

rived algebraic expectations, since at no point in the sequence of events from spermatocyte formation to emergence of the adult is there a biological basis for that operation, at least under generally accepted views as to what happens at each stage.

Let us consider the arguments against the possibilities that the experimental results are in fact based on some phenomenon found in the following stages: (1) zygotic, (2) gametic, and (3) meiotic.

1. *Zygotic:* This implies that the complementary gamete types are produced with equal frequencies but that the experimental results arise from an inviability of certain phenotypes, or mortality at an early stage because of some as yet unknown property of sperm of certain constitutions to kill eggs.

If this were the case, $A'B'$, with a recovered frequency of 0.191, is in fact produced with a frequency equivalent to that of its homologue AB, or 0.265, the difference being attributable to zygotic death, and, similarly, AB' should be adjusted to 0.428. Because the total is now greater than 1, these have to be adjusted to a total of 1 by dividing each value by the new total of 1.386, to give values of 0.191, 0.191, 0.309, and 0.309, respectively. In a mating of translocation males to normal females, only gametes of type AB produce zygotes that are not either lethal or semilethal, and this class should appear with a frequency of 19.1 per cent of the total zygotes. In an egg count, therefore, it should be found that no more than 19.1 per cent of the eggs laid by a female should give rise to progeny in this class, particularly since there is always some residual mortality that would tend to reduce the observed figure below expectation.

The results of the egg count show that there are too many adults recovered from the total of eggs laid, suggesting that zygote mortality is not the proper interpretation. Out of 920 eggs, 211 (22.9 per cent) carrying AB were recovered. A control run with females from the same stock (yellow) mated to wild-type (Canton-S) males gave 1,324 adults out of 1,416 eggs, or a hatchability of 93.5 per cent. This correction, applied to the experimental set, gives a frequency of 24.5 per cent recovery of AB. These results are significantly higher than the 19.1 per cent expected but agree closely with the 26.5 per cent expected if there is no zygote mortality.

Another argument against differential viability is that the percentages of recovery of AB (211) and $A'B'$ (174) in the corrected total number of eggs ($920 \times 0.935 = 860$) are 24.5 and 20.2 per cent, respectively, the ratio of these two values being close to the 26.5 and 19.1 figures from the attached-X crosses, despite the fact that the components are now recovered in the opposite sexes, with AB hemizygous in the attached-X matings but heterozygous in the matings to females with free X chromosomes.

2. *Gametic:* It seems, therefore, that the array of phenotypes derived in these experiments is probably an accurate indication of the array of functional sperm fertilizing eggs. We shall now consider the possibility that the discrepant results arise from some situation occurring between the formation of the spermatozoa and the fertilization of the egg. Sperm competition of the type found in plants and sometimes postulated to account for a possible deviation from 1:1 in primary sex ratio in humans, for instance, could hardly be expected in *Drosophila*, where the distance covered by the sperm between its storage place in the seminal receptacle of the female and its entry into the egg is less than that of the length of

the sperm tail. Gamete lethality must be considered a possibility. Certain of the sperm types may be differentially viable, but this supposition is at variance with the well-known capacity of sperm to function independently of their gene content, as shown by the recovery of unbalanced gametes with large deficiencies and duplications from translocation heterozygotes. In any case, sperm types AB and $A'B'$, occurring with frequencies of 0.265 and 0.191, respectively, are types that are genetically normal, i.e., one carries the full genetic complement of an X chromosome and the other that of a Y, yet one is found in these experiments 39 per cent more often than the other. Furthermore, if either of these types were to be held suspect because it contained an "abnormal" chromosome, it would be the AB gamete, which carries the B^S translocation, yet it is the more frequent of the two.

The exactness of the algebraic cross-check provides an argument against lethality of either gametes or zygotes, as such lethality is commonly conceived. Generally, in experiments with *Drosophila*, if a character depresses viability by a factor p_1 and another by a factor p_2, the combination of the two characters will not depress viability by the factor p_1p_2 with mathematical precision. Thus lethality would have to be of an unusual type, particularly since one of the characters with which a viability factor would have to be associated is the Y chromosome, ordinarily quite innocuous in single doses in all other kinds of experiments.

3. *Meiotic:* Another possibility to be considered, as a last resort, is that not all the products of spermatogenesis are functional and that we are observing a type of preferential segregation of certain of the components into those products that are functional. This view would be consistent with the probability formulation of the relationships of the gamete types. That preferential segregation could be the mechanism is suggested by the fact that, from each homologue pair, the one that is recovered more frequently is the smaller, a situation which, superficially at least, is similar to the phenomenon of the nonrandom disjunction in the female but which must differ in other respects, one of which is that genetically detectable exchange is a necessary requirement for nonrandom disjunction, whereas this type of exchange does not occur in the male.

A question to be considered is whether, if the latter is the case, this situation is a general property of *Drosophila* males or whether it is some peculiarity of the particular chromosome combinations used in this experiment. This cannot be answered at present and could be difficult to resolve conclusively, since it is only with homologues quite different from each other (implying that one, at least, is abnormal in some way) that this phenomenon might be observed.

Studies of spermatogenesis and spermiogenesis offer little support to the idea that some of the products of meiosis in the male are regularly nonfunctional. In *Drosophila* the best information is that each primary spermatocyte produces four functional sperm.[3] The same is true for studies made on mammals, although there is evidence for regular degeneration of some fraction of spermatocytes[4] and spermatogonia.[5] If, then, some fraction of the meiotic products are nonfunctional, the only supposition that is not inconsistent with cytological observations is that the time of such nonfunctioning is limited to the spermatozoal stage. This would not be ordinary gamete lethality, however, because it would have to be predetermined by the geometry of the meiotic divisions. Evidence on this possibility is clearly

difficult to obtain, as Hertwig[6] pointed out in 1890: "Wie aber will Jemand durch das Experiment beweisen, dass von den Millionen der in einem Hoden gebildeten Samenkörper ein jeder befruchtet?"

A few cases can be cited that are difficult to understand on the assumption that spermatogenesis proceeds in the accepted fashion but for which these experiments provide an interpretation. In the mouse, certain "*t*-alleles" are recovered from males with frequencies much greater than the 50 per cent expected, reaching values as high as 93 per cent. In a consideration of possible explanations for this result, Dunn and Suckling[7] state: "It must not be forgotten that some of the *t*-lethals may be connected with a chromosomal rearrangement, and this may introduce irregularities into spermatogenesis." It is conceivable, however, that spermatogenesis is not irregular in these cases but that the presence of a chromosomal rearrangement may serve to reveal a feature of normal spermatogenesis not detectable in ordinary experiments.

Furthermore, Ford, Carter, and Hamerton,[8] in a study of a translocation heterozygote in the mouse, confirm Snell's observation that the frequency of sperm producing viable offspring is about 60 per cent, a result inconsistent with their cytological observation that in 8–30 per cent of the spermatocytes there are rings of four, in the remainder bivalents; such a combination should produce about 40 or at the most 50 per cent of such sperm. This kind of discrepancy is similar to the one we have described for *Drosophila.*

In *Drosophila* experiments in which some element of doubt surrounds the validity of the observations because of the "complexity" of the chromosomes used, tests of a simpler situation should be made when possible. The results of the experiments with $T(1, 4)B^S$ suggest that if two unequal homologues are present in a male, the shorter may appear preferentially in the progeny, and the deviation from expectation may be so great that mortality can be excluded as the cause. D. L. Lindsley and L. Sandler have succeeded in acquiring confirmatory evidence to some extent in a less complex setup, and the authors are grateful to them for their permission to include some of their data. In measurements of the segregation of fragmented X chromosomes (small duplications) from an X-Y chromosome (a single chromosome combining the essential features of both X and Y chromosomes), they have found instances where the duplication has appeared more frequently than the X-Y homologue in the progeny and where egg counts clearly deny mortality. In one case (X-Y/duplication 179), out of 1,140 eggs, 797 of the emerging adults carried the fragment and only 292 the X-Y chromosome. In another case (X-Y/duplication 112), out of 1,792 eggs, there were 982 with the fragment and 583 with the X-Y chromosome; and in still a third (X-Y/duplication 167), out of 526 eggs, the numbers were 298 and 193. In each case, the difference between the egg count and the sum of the two adult classes represents egg mortality, and if the calculations are deliberately biased to minimize any discrepancy by assuming that all unhatched eggs belong in the class appearing least frequently in the progeny, the probabilities that these figures are random samples from a 1:1 ratio are, in each case, still less than 0.01. A reservation must be mentioned here, however. Not all fragmented X chromosomes studied show this effect, nor does the same fragment behave consistently. Lindsley and Sandler have evidence that segregation for an autosomal factor may alter the observed ratios. In any case, it can be stated with a high

degree of certainty that the 1:1 ratio of functional sperm carrying the two different homologues, demanded by the usual views of spermatogenesis and the gamete behavior of *Drosophila*, is not being obtained in these experiments, and some other explanation is necessary.

Summary.—Results from the study of segregation of the components of a translocation in the male of *Drosophila* cannot be interpreted easily on the basis of our present assumptions about the nature of spermatogenesis and gamete behavior. It is suggested that in these cases, and in others cited, not all products of spermatogenesis are functional and, further, that this condition may not be limited to these special cases but may be generally true, being revealed only when certain experimental conditions are achieved.

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[1] W. S. Stone, *Genetica*, **16**, 506–519, 1934.

[2] The authors wish to thank Dr. M. A. Kastenbaum, of the Mathematics Panel, Oak Ridge National Laboratory, who derived these gamete frequencies by the method of maximum likelihood.

[3] K. W. Cooper, *Biology of Drosophila* (New York: John Wiley & Sons, Inc., 1950), pp. 1–61.

[4] E. C. Roosen-Runge, *Z. Zellforsch. u. mikroskop. Anat.*, **41**, 221–235, 1955.

[5] E. F. Oakberg, *Am. J. Anat.*, **99**, 391–419, 1956.

[6] O. Hertwig, *Arch. mikroskop. Anat.*, **36**, 1–138, 1890.

[7] L. C. Dunn and J. Suckling, *Genetics*, **41**, 344–352, 1956.

[8] C. E. Ford, T. C. Carter, and J. L. Hamerton, *Heredity*, **10**, 284, 1956.

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*SHORTENING OF LIFE IN THE OFFSPRING OF MALE MICE EXPOSED TO NEUTRON RADIATION FROM AN ATOMIC BOMB**

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Introduction.—Only in recent years has evidence begun to accumulate that there are slight dominant deleterious effects of mutations formerly regarded as recessive.[1–4] The results to be reported here, and our earlier work on mice,[3, 5, 6] indicate that such effects may add up to an important part, perhaps the most important part, of the genetic hazards of radiation in man. The evidence from the earlier work on mice is that appreciable deleterious effects of radiation become manifest in the first-generation offspring. This evidence is of two kinds. First, work on radiation-induced mutations at specific loci in spermatogonia has shown

that among the recessive lethals, which comprise more than one-half of all the mutations recovered, many have dominant deleterious effects which, even for individual mutations, are sometimes large enough to be detected easily. Second, over-all population damage was found in the large numbers of animals that are raised as far as three weeks of age in the specific loci studies. In all such experiments carried out, the survival to three weeks of age is significantly lower in the offspring of irradiated males than it is in the controls. (It should perhaps be pointed out that neither of the above effects, nor the effect reported in this paper, is the result of what the geneticist usually refers to as "dominant lethals," which are major chromosomal aberrations that cause early death of embryos and which, as has been pointed out elsewhere,[5] are probably not an important hazard.)

Our earlier work that showed a significant effect on survival to three weeks of age in the offspring of irradiated males led us to expect that there would be measurable deleterious effects later in life. The data reported here show that such is indeed the case. These data furnish a third kind of evidence of first-generation damage and perhaps the most striking one. They were obtained as a by-product of another investigation, and they are not as extensive as we should like. However, they are the only data we have on this subject that were collected under the expensive and difficult conditions of a field test of a nuclear detonation. Furthermore, although the sample was small, it was sufficient to yield a statistically significant effect which appears to be large and, therefore, of general importance.

Materials and Methods.—The material used in the present longevity study was the by-product of an investigation of the relative effectiveness of neutrons from a nuclear detonation and from a cyclotron in inducing dominant lethals in the mouse.[7] In order to reduce the gamma component of the radiation to a proportion that would not appreciably interfere with the estimation of neutron effects, the animals were shielded with lead. The exposure chambers available were lead hemispheres of 7-inch wall thickness and 14-inch inside diameter. Young adult hybrid males, obtained by crossing inbred 101 strain females with inbred C3H strain males, were exposed inside the hemispheres placed at various distances from the detonation. Control males were placed in hemispheres two days before the detonation and for a length of time approximately the same as that required for the exposed animals. Further experimental details are described in the report of the earlier work.[7] One day and a half after the detonation, each male was placed with four adult untreated females of the same hybrid strain. At $18^1/_2$ days after irradiation each surviving male was placed with a new group of four females. Most of the females that became pregnant were killed at a late stage of gestation for the dominant-lethal study. However, since the number of pregnancies turned out to be more than adequate for the dominant-lethal experiment, several of the females were allowed to come to term. It was the offspring of some of these females that were saved for the longevity study described here. All these animals came from matings made from 19 to 23 days after irradiation. A few animals died before weaning, and these were not included in the data reported here. At weaning age the sexes were separated and the animals grouped, so far as possible, six to a cage. They were kept in the same grouping throughout their lifespan. They were checked at least twice weekly for deaths. Only one animal died at less than one year of age, indicating that the conditions under which the animals were kept were good.

The total (neutron plus gamma radiation) dose inside each lead hemisphere was measured, as described in the earlier publication,[7] by means of "tissue-equivalent" ion chambers designed for this purpose at short notice.[8] Subsequently, extensive testing and recalibration of these chambers[9] has led to a revision of the original dose estimates. The doses reported in the present publication are the revised estimates. As was reported earlier,[7] attempts to measure the gamma component of the radiation by means of film dosimeters left a large uncertainty as to the size of this factor. Later tests have been made in which both ionization chambers and chemical dosimeters were used to measure the gamma component inside the lead hemispheres when these were exposed to fission neutrons. According to the latest information,[10] these tests indicate that the gamma-radiation exposure in our experiment was almost certainly less than 10 per cent of the total dose.

Results.—The median and mean lengths of life, together with the number of animals, for each dose group are given in Table 1. An analysis of variance showed that neither grouping in cages nor sex had a significant effect on length of life. It seems likely that larger samples would show some effect of both of these factors, but as there was no significant effect of them in the present experiment, the data were pooled.

TABLE 1

LENGTH OF LIFE IN THE OFFSPRING OF MALE MICE EXPOSED TO NEUTRON RADIATION 19–23 DAYS BEFORE MATING

(Deaths before Weaning Age Excluded)

Total Dose to Parent (rep)*	No. of Offspring	Median Length of Life of Offspring (Days)	Mean Length of Life of Offspring (Days)
0	103	823	792
31	50	741	754
71	5	717	699
118	22	739	723
136	8	666	688
186	2	756	756

* Includes some gamma radiation, estimated to be less than 10 per cent of the total dose.

To test whether there was a significant effect of radiation on the length of life of the offspring, the means were fitted to a straight line by the method of weighted least squares. This gives an intercept of 786 days and a slope of -0.609 ± 0.238. Since the residual variance is less than the within-subclass mean square, there is no evidence of nonlinearity over the dose range tested. Even if the true shape of the curve is nonlinear, it will be conservative, in making the test of significance, to assume linearity. The larger mean square was used to compute the variance of the slope, and a two-sided t-test shows that the slope differs significantly from zero at the 1 per cent level. If one is willing to accept a one-sided t-test as more appropriate, the significance level is 0.5 per cent. Thus there is strong evidence of shortening of life in the offspring of the exposed males.

Discussion.—It is noteworthy that a significant shortening of life was detected in spite of the small sample and the considerable genetic variability that must have been present in a population that was the F_2 of a cross between inbred strains. Furthermore, the weighted mean dose received by the exposed fathers was only moderate, being less than one-sixth of the 30-day median lethal dose as measured from other animals of the same strain exposed under the same conditions at distances

closer to the same detonation. While it is true that certain features of the experiment, which will be discussed later, tended to maximize the shortening of life, nevertheless the result observed appears to be so large that it seems quite possible that shortening of life is an effect that might be detectable in studies of the offspring of exposed parents in human populations.

In view of the lack of information on this subject, and specifically the fact that no data of this nature were ready for consideration prior to the writing of the 1956 report of the National Academy of Sciences Committee on Genetic Effects,[11] it is desirable to consider what the present data might indicate when they are extrapolated to man. Taking the estimate obtained from the curve fitted to the mouse data, and assuming that the shortening of life in man would be proportional to this, gives, on the basis of a 70-year length of life in man, the figures shown in Table 2. It should be kept in mind that the results were obtained from neutron

TABLE 2

SHORTENING OF LIFE IN THE OFFSPRING OF FATHERS EXPOSED TO NEUTRON RADIATION 19–23 DAYS BEFORE MATING

OBSERVED RESULT IN THE MOUSE AND EXTRAPOLATION TO MAN

(Deaths before Weaning Age Excluded)

	Mouse	Man
Point estimate	0.61 day/rep to father	20 days/rep to father
Lower 95 per cent confidence limit	0.14 day/rep to father	5 days/rep to father
Upper 95 per cent confidence limit	1.07 day/rep to father	35 days/rep to father

irradiation. The relative biological effectiveness of neutrons for this effect is not known, but it seems likely, from other data on mutations, that gamma and X-radiation would be less effective than neutrons. It should also be emphasized that the effect observed here is probably a maximum one, since the offspring were obtained from matings made between 19 and 23 days after irradiation. Our data from experiments on mutations at specific loci[12] indicate that the sperm utilized in matings made within this time interval would have been derived from cells in a sensitive stage of gametogenesis at the time of irradiation. From approximately two to four times as many mutations are recovered from this stage as from the spermatogonial stage, which is the important one so far as radiation hazards in man are concerned.[6] It is also possible that the spectrum of mutations from irradiated spermatogonia would be qualitatively different and, conceivably, less effective in shortening life. However, there is no direct evidence of this, whereas there is evidence from our specific loci studies that some mutations induced in spermatogonia have, even individually, a dominant effect on length of life that is detectable. To summarize this paragraph: it should be remembered that the estimates given in Table 2 are based on neutron irradiation of a post-spermatogonial and sensitive stage in gametogenesis and that X- or gamma irradiation of spermatogonia would almost certainly produce a smaller effect.

Another way of considering the magnitude of the observed result, so far as its human implications are concerned, is to compare the shortening of life in the offspring of irradiated fathers with that in the irradiated individuals themselves. The data on shortening of life of the males exposed to this same detonation will be presented in detail elsewhere. Briefly, the percentage shortening of life of these animals, based on 24 controls and 128 exposed animals, is 0.078 per cent per rep.

The present data, expressed in the same form, give 0.077 per cent shortening of life in the offspring for each rep received by the father, that is, approximately as much effect as on the exposed individuals. Thus the best estimate from our present data is that, for neutron irradiation of the sensitive stages in spermatogenesis, the shortening of life in the offspring of irradiated males will be similar in magnitude to that in the exposed individuals. Again, the effect from irradiation of spermatogonial stages would probably be less. Whether the *ratio* of effect in offspring to effect in exposed individuals will be different for X- and gamma rays from that observed for neutrons will, of course, depend on whether the relative biological effectiveness of neutrons is different for the effect on the offspring and the effect on the exposed individuals. Present, incomplete data on these points give no grounds for expecting that the ratio of effect in offspring to effect in exposed individuals will be less for X-rays than for neutrons. Weighing the evidence reported here, and making some allowance for the many uncertainties, it seems reasonable to predict that, even under the conditions of radiation exposure in man, shortening of life in the offspring of irradiated fathers will be between 10 and 100 per cent of the shortening of life in the exposed individuals themselves. It should be remembered that this excludes an additional effect on the offspring, namely, as measured in the mouse, death before weaning age. Also, and more important, since the shortening of life is probably the result of mutations with slight dominant effects, the damage would not end with the first-generation offspring but would, to a certain, and probably large, degree, be transmitted to later generations.

Summary.—Length of life in the offspring of male mice exposed to moderate doses of neutron radiation from a nuclear detonation is shortened by 0.61 day for each rep received by the father over the dose range tested. This figure excludes death before weaning age. The 95 per cent confidence limits are 0.14 and 1.07 days per rep. Extrapolating to a proportional shortening of life in man gives 20 days per rep received by the father as the point estimate and 5 and 35 days as the 95 per cent confidence limits. The offspring were obtained from matings made from 19 to 23 days after irradiation and therefore represent the effect of irradiation on germ cells in a post-spermatogonial and sensitive stage of gametogenesis. It is probable that irradiation of spermatogonia (the stage that is important from the point of view of human hazards) would give a somewhat smaller effect. However, since the present data show an effect on the offspring which is as large as the shortening of life in the exposed individuals themselves, it seems likely that, even when allowance is made for the conditions of human radiation exposure, shortening of life in the immediate descendants will turn out to be of a magnitude that will warrant serious consideration as a genetic hazard in man.

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[1] C. Stern and E. Novitski, *Science*, **108**, 538–539, 1948.
[2] H. J. Muller, *J. Cellular Comp. Physiol.*, **35**, suppl. 1, 205–210, 1950.
[3] W. L. Russell, *Cold Spring Harbor Symposia Quant. Biol.*, **16**, 327–336, 1951.
[4] C. Stern, G. Carson, M. Kinst, E. Novitski, and D. Uphoff, *Genetics*, **37**, 413–449, 1952.
[5] W. L. Russell, in *Radiation Biology*, Vol. I, ed. A. Hollaender (New York: McGraw-Hill Book Co., 1954), chap. xii.
[6] W. L. Russell, *Proceedings of the International Conference on the Peaceful Uses of Atomic Energy*, **11** (New York: United Nations, 1956), 382–383, 401–402.
[7] W. L. Russell, L. B. Russell, and A. W. Kimball, *Am. Naturalist*, **88**, 269–286, 1954.
[8] C. W. Sheppard and E. B. Darden, appendix to J. S. Kirby-Smith and C. P. Swanson, *Science*, **119**, 42–45, 1954.
[9] C. W. Sheppard, M. Slater, E. B. Darden, Jr., A. W. Kimball, G. J. Atta, C. W. Edington, and W. K. Baker, *Radiation Research* (in press).
[10] G. S. Hurst, personal communication.
[11] *The Biological Effects of Atomic Radiation: Summary Reports* (Washington: National Academy of Sciences, National Research Council, 1956).
[12] W. L. Russell, *USAEC Unclassified Report ORNL-2155* (Washington: Office of Technical Services, Department of Commerce, 1956).

CORRELATED SELECTION FOR MOTILITY AND SEX-INCOMPATIBILITY IN ESCHERICHIA COLI K12

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Sexual fertility in *Escherichia coli* is influenced by a number of factors, environmental and genetic.[1] One of the latter is *F*, which is diagnosed by the following rules of compatibility: $F^+ \times F^+$ and $F^+ \times F^-$ are fertile, but $F^- \times F^-$ is sterile. The wild-type K12 strain is F^+, but "self-incompatible" F^- mutants have appeared sporadically in laboratory cultures. Among the genetic elements of *E. coli*, *F* is remarkable for its high contagiousness: F^- cells become F^+ when the two types are grown in mixed culture. Furthermore, the progeny of $F^+ \times F^-$ crosses are uniformly F^+. Therefore, a reliable method of obtaining F^- derivatives of various stocks would be desirable as a technical help[2] as well as for its possible clarification of the nature of *F*.

In the course of immunogenetic studies of *E. coli*, various strains were *motilized*, that is, passed through semisolid agar to select for the highest motility and development of flagellar antigens. Many of the selected clones proved to be F^-. The experiments reported here indicate that this is a result of a selective advantage of F^- mutants rather than a direct induction of them by the technique.

Experimental.—The motilization medium (NGA) consists of nutrient broth plus 8 per cent gelatin and 0.4 per cent agar.[3] Comparable results were obtained in the same medium without gelatin. In these media, nonmotile bacteria grow only at the site of inoculation, while motile bacteria eventually swarm through the medium. After an initial lag, swarms appear from which faster swarms may arise in turn. The fastest rate of swarming observed on this medium (at 37° C.) was 3 mm/hr. The succession of faster swarms upon subcultures on fresh NGA medium,

together with preliminary genetic analyses, indicate that motility is under polygenic control.

For motilization, the bacteria are inoculated at the top of a culture tube containing a column of NGA about 6 cm. deep. After swarmers reach the bottom, the top 5-cm. layer is melted off and a loopful from the bottom layer streaked on nutrient agar.[4] Single-colony cultures are tested for their *F* status by established methods.[5]

Many F^+ substrains of *E. coli* K12 have been motilized and tested for their *F* status. About half of several hundred of these motilizations have given F^- clones after a single tube passage. A few dozen cultures, which had not yielded F^- progeny at first, were examined further, and it was always possible to obtain an F^- culture after a few additional passages.

Most of the isolates so obtained are identical with standard F^- cultures in the following respects: (1) their compatibility pattern, which is clonally stable; (2) their reconversion to F^+ when grown with F^+ cells; (3) the clonal stability of the reconverted F^+; and (4) the production of F^- derivatives when these are again motilized. Self-incompatible mutants were also obtained from another mating type, *Hfr*, including some novel types. These included refractory F^- (not reconvertible to F^+ by mixed culture) and stocks of intermediate fertility; they require further study.

The incompatible phenotype has also been secured by growing F^+ cultures with heavy aeration. However, this is an F^- *phenocopy*. Unlike the F^- mutants, these incompatible cells produce only compatible F^+ clones when they are grown out under standard conditions. In contrast, some motilized F^- substrains have been subcultured for four years without manifesting any change in this trait. The motilization technique has been applied successfully to all of the principal crossing stocks in use in this laboratory.[2, 5, 6]

There remains the problem whether motilization induces or selects the F^- mutants. During the procedure, the bacteria are continuously exposed to fresh medium and are therefore growing rapidly under chemostatic conditions. The conditions might be analogous to those for the attenuation of kappa in *Paramecium*.[7] To explore this analogy, various F^+ cultures were grown in chemostats[8] in a defined medium. Although the first run did give an F^- isolate (perhaps by an accident of periodic selection), no further trials were successful, nor was a trial in which a dilute *Hfr* culture was transferred for 100 fissions at intervals of 4 hours (10 fissions). These results were therefore indecisive.

F^+ cultures generally, including those obtained by reconversion of motilized F^- isolates, are not strikingly less motile than their F^- counterparts. Different stocks show wide differences in their initial motility, independently of their *F* status. However, the competitive status of F^+ and F^- cells could be assessed directly in NGA, as follows. To minimize genetic heterogeneity, closely related stocks were developed. A Gal^- (galactose- negative) F^+ stock was motilized, and a Gal^-F^- isolate recovered. From this, Gal^+F^-, Gal^-F^+, and Gal^+F^+ derivatives were secured by reverse mutation (selected on EMB galactose agar), *F* reconversion, or both. Pairs of cultures were then mixed in equal proportions and inoculated in broth and in NGA plates. After overnight incubation, the broth cultures were plated on EMB galactose agar for relative counts of Gal^+:Gal^-. The NGA plates were incubated for 24 hours, at which time samples were cored out from swarms

(about 7 cm. from the point of inoculation), dispersed in water, and plated likewise. As shown in Table 1, the Gal^+ marker is not entirely neutral and has an advantage

TABLE 1*

COMPETITION OF Gal^+/Gal^- AND F^+/F^-

Mixture		Inoculum Ratio		Ratio after Passage in Broth		Ratio after Passage in NGA		Samples from Replicate NGA Passages		
Gal^+	Gal^-	Gal^+	Gal^-	Gal^+	Gal^-	Gal^+	Gal^-	Pure Gal^+	Pure Gal^-	Mixed $Gal^+,^-$
F^+	F^+	246	194	226	186	393	4	0	0	10
F^+	F^-	229	155	370	154	0	595	0	9	1
F^-	F^-	187	142	355	127	606	400	0	0	10
F^-	F^+	247	240	317	193	310	0	7	0	1

* The counts are from two plates each of EMB galactose agar. The total density of the inoculum was about 10^9/ml. Its growth in the broth passage would correspond to about 10 fissions. In NGA this would be more variable, but some cells at the frontier might have a history of up to 50 fissions if they were not inhibited by metabolic products diffusing from the population behind them. In the replicate NGA passages, one sample from each motilization plate was streaked on EMB galactose agar.

over Gal^-, but this is superimposed on, and can be overridden by, an advantage of F^- over F^+ during passage in motility agar. The consistency of the selective differences was shown by replicate tests with the same mixtures. However, this experimental design still suffers from the possibility of random mutational drift (with respect to motility polygenes) in the few isolated clones.

These findings encouraged attempts to measure differences in the motility of comparable F^- and F^+ isolates. Exponential phase cultures of a motilized isolate of W945 ($T^-L^-Th^-Gal_2^-F^-$) and of K12 (Gal^+F^+) were mixed in broth at a ratio of 1:10. After 1 hour's incubation, when about half the F^- cells should have been converted to F^+, the mixture was plated on EMB galactose agar. Gal^- colonies were picked to broth. These cultures were tested for their compatibility by appropriate crosses and, at the same time, loopfuls were inoculated into NGA tubes. The progress of the swarm front was measured after 16 hours' incubation. Twenty-four isolates proved to be still F^-; seventeen were F^+. As shown in Figure 1, there

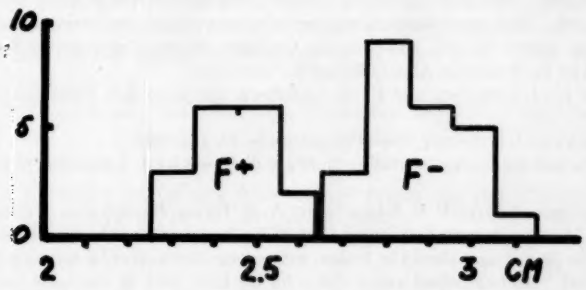

Fig. 1.—Relative motility of F^+ and F^- isolates. *Ordinate:* frequency. *Abscissa:* progression of swarm front after 16 hours' incubation of NGA tubes.

was a clear assortment of these classes in respect to motility ($t_{39} = 13$; $p < .01$), although the intraclass variation (random drift) is equally plain. The same experiment has been repeated with other F^- stocks which were inherently more variable, with similar if less striking results.

Discussion.—The multistep response to selection suggests that motility in bacteria is under polygenic control, as it is also in *Drosophila.*[9] Bacterial flagella are also subject to oligogenic mutations[3] which, however, are not involved in these *E. coli* stocks. On this polygenic background, the F^- mutation appears to have a further effect in augmenting motility, which is a sufficient explanation of the correlated selection. This assumes the occasional occurrence of spontaneous F^- mutations. Whether the sporadic mutants already cited,[5, 6] were "spontaneous" or related to incidental mutagenic treatments would be different to verify without more convenient methods of detecting rare F^- mutants. The same obstacle makes it difficult to rule out further direct effects of the motilization procedure on the rate of F^- mutation.

An effect of F status on motility is not unexpected if the compatibility phenotype is to be associated with the cell surface. F^- cells are reportedly more electronegative[10] and hence less readily agglutinated in saline and certain antiserums than F^+. Unfortunately, *E. coli* K12 is so "rough" as to be particularly unsuited to serological studies by agglutination techniques. Further work is therefore needed to test such speculations as the identification of a hypothetical F substance with a surface layer that mechanically impedes motility. Immunogenetic studies with more suitable strains are in progress.

The inference that motilization has only a selective effect limits its interest for the general problems of the nature of F, whether it is a nuclear or extranuclear element, and the manner of its transmission in reconversion experiments. On this hypothesis, the procedure has no bearing on the mutational event $F^+ \rightarrow F^-$, but only on its secondary phenotypic effects.[11]

Summary.—In the course of selection for high motility, F^+ (sexually compatible) cultures of *E. coli* frequently produce F^- (self-incompatible) clones. This technically useful effect can be explained by a higher motility of F^- genotypes, superimposed on a polygenic control of motility.

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[1] See review by J. Lederberg and E. M. Lederberg, *Symposia Soc. Development and Growth*, **14**, 101, 1956.

[2] T. C. Nelson and J. Lederberg, these PROCEEDINGS, **40**, 415, 1954.

[3] The history and application of this medium are discussed by J. Lederberg, *Genetics*, **41**, 845, 1956.

[4] This simple procedure (cf. P. R. Edwards and W. H. Ewing, *Identification of Enterobacteriaceae* [Minneapolis, Minn.: Burgess Publishing Co., 1955]) has perils for the uninitiated. To avoid an explosion, the plug of agar should be broken with a long wire loop or by spinning the tube while it is being heated. An improvised socket clamp for the tube, with an extension rod to rotate between the fingers is helpful. Many complicated methods, including U-tubes and concentric tubes, for reaching the same ends have been described, and NGA plates may also be used where convenient.

[5] J. Lederberg, L. L. Cavalli, and E. M. Lederberg, *Genetics*, **37**, 720, 1952.

[6] W. Hayes, *Cold Spring Harbor Symposia Quant. Biol.*, **18**, 75, 1953, has mentioned an F^- mutant stock he received from C. C. Spicer. Dr. Spicer has informed us, in retrospect, that the parent culture had in fact been motilized and put in cold storage before being sent to Dr. Hayes.

[7] J. R. Preer, Jr., *Genetics*, **33**, 349, 1948.

[8] A. Novick and L. Szilard, *Cold Spring Harbor Symposia Quant. Biol.*, **16**, 337, 1951; B. Rot-

man, *J. Bacteriol.*, **70**, 485, 1955. We are indebted to these authors for their help with these experiments.

[9] H. L. Carson, *Genetics*, **41**, 636, 1956 (abstr.).

[10] G. A. Maccacaro and R. Comolli, *J. Gen. Microbiol.*, **15**, 121, 1956.

[11] Y. Hirota, *Nature*, **178**, 92, 1956, has reported that exposure of F^+ cells to Co^{++} solutions induces the formation of F^- types in certain strains.

A UNIFIED THEORY OF GENERAL RELATIVITY OF GRAVITATION AND ELECTROMAGNETISM. IV

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This paper derives in a somewhat different manner the final results of my third paper.[1] The theory is based upon a symmetric tensor g_{ij}, a coefficient of connection Γ^i_{jk}, and an electromagnetic vector φ_i, in terms of which there is the skew-symmetric electromagnetic field tensor

$$F_{ij} = \varphi_{i,j} - \varphi_{j,i} = \varphi_{i;j} - \varphi_{j;i}. \tag{1}$$

Here, and throughout the paper, a comma followed by an index denotes the derivative with respect to x with this index, and a semicolon followed by an index denotes the corresponding covariant derivative in terms of the Christoffel symbols in g_{ij}.

From equation (1) it follows that

$$F_{ij,k} + F_{jk,i} + F_{ki,j} = 0, \tag{2}$$

from which one has

$$F_{ij;k} + F_{jk;i} + F_{ki;j} = 0. \tag{3}$$

It being required that the determinant g of g_{ij} be different from zero, and in fact negative, a contravariant symmetric tensor g^{ij} is defined by $g^{ij}g_{ik} = \delta^j_k$, where δ^j_k is the Kronecker delta, and where a repeated superscript and subscript, as i in this case, means the sum as the index takes the values 1 to 4. This notation holds throughout the paper.

For any coefficients of connection Γ^i_{jh}, symmetric in j and h, one has

$$\Gamma^i_{jh} = \left\{ {i \atop jh} \right\} + a_{jh}{}^i, \tag{4}$$

where $a_{jh}{}^i$ is a tensor, as follows from the equation for the Christoffel symbols $\left\{ {i \atop jh} \right\}$ and Γ^i_{jh} separately in two co-ordinate systems.[2]

We take

$$a_{jh}{}^i = g_{jh}\lambda^i,$$

where λ^i is a vector, and have

$$\Gamma^i_{jh} = \left\{ {i \atop jh} \right\} + g_{jh}\lambda^i. \tag{5}$$

For coefficients of connection Γ^i_{jh} there is the tensor[3]

$$\Gamma^i_{jhl} = \Gamma^i_{jl,h} - \Gamma^i_{jh,l} + \Gamma^k_{jl}\Gamma^i_{kh} - \Gamma^k_{jh}\Gamma^i_{kl}.$$

Since the covariant derivative of g_{jh} is equal to zero, for Γ^i_{jh} given by equation (5) one has

$$\Gamma_{jh} = \Gamma^i_{jhi} = R_{jh} + \lambda_{j;h} + \lambda_j\lambda_h - g_{jh}(\lambda^i{}_{;i} + \lambda^i\lambda_i),$$

where $R^i{}_{jhl}$ is the Riemann tensor.[4] Contracting for i and l, one has

$$\Gamma^i{}_{jhi} = R^i{}_{jhi} + g_{ji}\lambda^i{}_{;h} - g_{jh}\lambda^i{}_{;i} + \lambda^i(\lambda_h g_{ji} - \lambda_i g_{jh}),$$

where R_{jh} is the Ricci tensor.[5]

We require that

$$\Gamma_{jh} = 0.$$

For the skew-symmetric part of Γ_{jh} equated to zero there is

$$\lambda_{j;h} - \lambda_{h;j} = 0, \tag{6}$$

and for the symmetric part of Γ_{jh} equated to zero one has

$$R_{jh} = -{}^1/_2(\lambda_{j;h} + \lambda_{h;j}) - \lambda_j\lambda_h + g_{jh}(\lambda^i{}_{;i} + \lambda^i\lambda_i). \tag{7}$$

We put

$$\lambda_{j;h} = -\lambda_j\lambda_h + b_{jh}, \tag{8}$$

where b_{jh} is a symmetric tensor in consequence of equation (6). For equations of the form (8), equation (7) becomes

$$R_{jh} = -b_{jh} + g_{jh}b, \tag{9}$$

where

$$b = g^{ji}b_{ji} = b^i{}_i.$$

For a vector λ_i there is the equation[6]

$$\lambda_{i;hl} - \lambda_{i;lh} = \lambda^j R_{jihl}.$$

For equations of the form (8) this equation is

$$\lambda^j R_{jihl} = \lambda_i b_{ih} - \lambda_h b_{il} + b_{ih;l} - b_{il;h}.$$

When this equation is multiplied by g^{il} and contracted for i and l, the result may be written

$$-\lambda R_{jh} = \lambda^i b_{ih} - \lambda_h b + b^i{}_{h;i} - b,h,$$

since the covariant derivative of g^{il} is equal to zero.

From equation (9) one has

$$\lambda^j R_{jh} = -\lambda b_{ih} + \lambda_h b.$$

When this equation is added to the preceding one, there results the equation

$$b^i{}_{h;i} - b_{,h} = 0. \tag{10}$$

Consider equation (9) written in the form

$$R^i{}_h = -b^i{}_h + \delta^i{}_h b,$$

where $\delta^i{}_h$ is the Kronecker delta. One has

$$R = R^i{}_i = 3b.$$

For these expressions for $R^i{}_h$ and R the identity[7]

$$R^i{}_{h;i} - {}^1/_2 R_{,h} = 0$$

imposes the condition

$$b^i{}_{h;i} + {}^1/_2 b_{,h} = 0,$$

since the covariant derivative of $\delta^i{}_h$ is equal to zero. From this result and (10) one has

$$b^i{}_{h;i} = 0, \qquad b_{,h} = 0. \tag{11}$$

We take

$$b_{jh} = -F_j{}^i F_{hi} + {}^1/_4 g_{jh} F_{ik} F^{ik}. \tag{12}$$

One has

$$b = g^{jh} b_{jh} = -F^{hi} F_{hi} + F_{ik} F^{ik} = 0,$$

so that the second of equations (11) is satisfied. From equation (12) one has

$$b^i{}_h = -F^{ij} F_{hj} + {}^1/_4 \delta^i{}_h F_{jk} F^{jk},$$

from which one obtains

$$\begin{aligned} b^i{}_{h;i} &= -F^{ij}{}_{;i} F_{hj} - F^{ij} F_{hj;i} + {}^1/_4 (F_{jk} F^{jk})\,;\, h \\ &= -F^{ij}{}_{;i} F_{hj} + F^{jk} (F_{hj;k} + {}^1/_2 F_{jk;h}). \end{aligned} \tag{13}$$

By means of equations of the form (3) one has

$$\begin{aligned} F_{hj;k} + {}^1/_2 F_{jk;h} &= {}^1/_2 (F_{hj;k} + F_{hj;k} + F_{jk;h}) \\ &= {}^1/_2 (F_{hj;k} + F_{hk;j}). \end{aligned}$$

Hence equation (13) reduces to

$$b^i{}_{h;i} = -F^i{}_{j;i} F_h{}^j,$$

from which it follows that the first of conditions (11) is satisfied in accordance with the Maxwell equation

$$F^i{}_{j;i} = 0.$$

Since b is equal to zero, it follows from equations (9) and (12) that

$$R_{jh} = F_j{}^i F_{hi} - {}^1/_4 g_{jh} F_{ik} F^{ik}. \tag{14}$$

This is the equation proposed by Einstein.[8] In proposing it, he made the following statement: "This inclusion of the theory of electricity in the scheme of the general theory of relativity has been considered arbitrary and unsatisfactory by many theoreticians.... A theory in which the gravitational field and the electromagnetic field do not enter as logically distinct structures would be much preferable."

[1] Cf. these PROCEEDINGS, **42**, 878–881, 1956.
[2] L. P. Eisenhart, "Non-Riemannian Geometry," *Am. Math. Soc. Pubs.*, No. 8, p. 9.
[3] *Ibid.*, p. 5.
[4] L. P. Eisenhart, *Riemannian Geometry* (Princeton, N. J.: Princeton University Press, 1949), p. 19.
[5] *Ibid.*, p. 21.
[6] *Ibid.*, p. 30.
[7] *Ibid.*, p. 82.
[8] A. Einstein, *The Meaning of Relativity* (2d ed.; Princeton, N. J.: Princeton University Press, 1945), p. 98. In the third edition (1950), the fourth edition (1953), and the fifth edition (1955) p. 98 of the second edition continues as p. 98.

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*INTEGRATION OVER HILBERT SPACE AND OUTER EXTENSIONS**

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With a view toward possible application to the quantum theory of fields, various processes of integration over Hilbert space have been introduced.[1] In this note another such process is considered, and a sketch is given of various notions and results which manifest themselves. For any real separable Hilbert space $\mathfrak{H}$, let $\mathfrak{R}$ denote the collection of finite-dimensional subspaces E of $\mathfrak{H}$. In each $E \in \mathfrak{R}$, we introduce the Gaussian measure μ_E defined by

$$d\mu_E = \sqrt{\left(\frac{a}{\pi}\right)^n}\, e^{-a(x_1^2 + \ldots + x_n^2)}\, dx_1 \ldots dx_n, \qquad a > 0,\ n = \dim E, \tag{1}$$

where $x_1, \ldots, x_n$ is a particular co-ordinatization of E. Because of the unitary invariance of the Gaussian measure, the measure thus defined is in fact independent of the particular co-ordinate system for E.

A function $f(x)$, defined over $\mathfrak{H}$, is called a *cylinder function* if there exists at least one $E \in \mathfrak{R}$ such that $f(P_E x) = f(x)$ for all x; here P_E is the orthogonal projection onto E. If so, E is referred to as a "base space for f," and $f(x)$ is said "to have a base in E." We next consider, for any $\alpha \geq 1$, the collection $\mathfrak{C}_\alpha^E$ of cylinder functions $f(x)$ having a base in E, and such that $|f(x)|^\alpha$ is integrable over E with respect to the measure μ_E. It is then easily seen that the union $\mathfrak{C}_\alpha = \bigcup_{E \in \mathfrak{R}} \mathfrak{C}_\alpha^E$ is a linear space. Also, it is easily seen that for $f(x) \in \mathfrak{C}_\alpha^E$, the integral

$$I_E(|f|^\alpha) = \int_E |f(P_E x)|^\alpha\, d\mu_E \tag{2}$$

is the same for all $E \in \mathfrak{R}$ in which $f(x)$ has a base. For such cylinder functions we define the "Hilbert space integral,"

$$I_{\mathfrak{H}}(|f|^\alpha) = I_E(|f|^\alpha). \tag{3}$$

A sequence of projections (P^n) with ranges in $\mathfrak{R}$ is said to be *complete* if, for all $x \in \mathfrak{H}$, $P^n x \to x$ as $n \to \infty$. In particular, we shall consider "basic systems" of projections, (P^n), corresponding to an orthonormal basis $g_1, g_2, \ldots$, of $\mathfrak{H}$, wherein, for $x \in \mathfrak{H}$, $x = \sum_{i=1}^{\infty} x_i g_i$, $P^n x = \sum_{i=1}^{n} x_i g_i$. For a given $\alpha \geq 1$, we form the linear space

$\mathcal{C}_\alpha^{(P^n)} = \bigcup_n \mathcal{C}_\alpha^{P^n}$, and introduce in $\mathcal{C}_\alpha^{(P^n)}$ the norm $\|f\|_{(P^n)} = \{I_{\mathfrak{X}}(|f|^\alpha)\}^{1/2}$. In this norm, $\mathcal{C}_\alpha^{(P^n)}$ may be completed, and the norm extended, to a linear space $\mathfrak{L}_\alpha^{(P^n)}$. The elements of $\mathfrak{L}_\alpha^{(P^n)}$ are "ideal functions" associated with sequences of cylinder functions of $\mathcal{C}_\alpha^{(P^n)}$ which are Cauchy sequences in the norm $\| \ \|_{(P^n)}$.

In connection with these notions, we look for a natural "identification" of the ideal functions of $\mathfrak{L}_\alpha^{(P^n)}$ and $\mathfrak{L}_\alpha^{(Q^m)}$, for two basic systems (P^n) and (Q^m). More precisely, for the unitary transformation corresponding to the change from the basis of (P^n) to that of (Q^m), we seek a norm preserving "transformation" of $\mathfrak{L}_\alpha^{(P^n)}$ into $\mathfrak{L}_\alpha^{(Q^m)}$. Such "transformations" can be constructed and in fact are easily obtained, from the following:

THEOREM A.[2] *Let $f(x) \in \mathcal{C}_\alpha^E$ for some E, $\alpha \geq 1$; and let (R^n) be any complete sequence of projections such that $R^n\mathfrak{X} \in \mathfrak{R}$. Then*

$$\lim_{m,\, n \to \infty} I_{\mathfrak{X}}(|f(R^n\cdot) - f(R^m\cdot)|^\alpha) = 0. \tag{4}$$

Theorem A holds also if the measures μ_E, as defined in equation (1), are "complex" Gaussian measures, that is, if the parameter a is a complex number with positive real part.

For any fixed $\alpha \geq 1$, we define a (proper) function over $\mathfrak{X}$ to be *invariant* if and only if (i) for all basic systems (P^n),

$$\{f(P^n x)\} \in \mathfrak{L}_\alpha^{(P^n)};$$

and (ii) for any two basic systems (P^n), (Q^m),

$$\lim_{m,\, n \to \infty} I_{\mathfrak{X}}(|f(P^n\cdot) - f(Q^m\cdot)|^\alpha) = 0.$$

For such an $f(x)$, we may define

$$\int_{\mathfrak{X}} |f(x)|^\alpha \, d\mu_{\mathfrak{X}} = \lim_{n \to \infty} I_{\mathfrak{X}}(|f(P^n\cdot)|^\alpha).$$

The statement of Theorem A then implies that *integrable cylinder functions are invariant.*

To attain total additivity of the measure, which is lacking in Hilbert space, we must extend this space. Corresponding to a co-ordinatization of $\mathfrak{X}$, given with the aid of a basic system (P^n), there is an imbedding of $\mathfrak{X}$ in an unrestricted sequence space $W^{(P^n)}$, which we call *a basic corona* of $\mathfrak{X}$. The Kolmogoroff extension theorem provides a totally additive Gaussian measure $\mu_{W^{(P^n)}}$ in $W^{(P^n)}$ such that for quasi-intervals J of $W^{(P^n)}$ we have $\mu_{\mathfrak{X}}(J \cap \mathfrak{X}) = \mu_{W^{(P^n)}}(J)$. The following theorem describes the relationship between basic coronas arising from two basic systems.

THEOREM B.[3] *For any two basic systems (P^n) and (Q^m) there exists, apart from sets of measure zero outside $\mathfrak{X}$, a 1-1 measure preserving transformation between the basic coronas $W^{(P^n)}$ and $W^{(Q^m)}$, which leaves the elements of $\mathfrak{X}$ fixed.*

The measure preserving transformation in the above theorem is constructed so that, after suitably ignoring certain sets of measure zero outside $\mathfrak{X}$, weak convergence is preserved. This particular mapping, referred to as the *natural transfer* between $W^{(P^n)}$ and $W^{(Q^m)}$, is in fact characterized by these properties.

We mention an immediate consequence of Theorem B. Consider any countable collection of basic systems (P_i^n), $i = 1, 2, \ldots$, and identify elements which corre-

spond under natural transfer. Thus there results a space $W \supset \mathfrak{C}$, called a *corona* of $\mathfrak{C}$, such that all projections P_i^n are defined on W, $P_i^n W \subset \mathfrak{C}$. A totally additive Gaussian measure μ_W may be introduced in W such that $\mu_W(J) = \mu_{\mathfrak{C}}(J \cap \mathfrak{C})$ for any quasi-interval J of W.

The following result concerning invariant functions is a consequence of Theorem A, the Fischer theorem, the continuity of the Gaussian distribution, and the construction described above.

THEOREM C. *Let $f(x)$ be an invariant proper function over $\mathfrak{C}$, for $\alpha = 1$; then there exists a function $\hat{f}(x)$, defined on W, such that*

$$\hat{f}(P_i^n x) = f(P_i^n x), \qquad \text{all } i, n;$$

and

$$\lim_{n \to \infty} \int_W |\hat{f}(x) - \hat{f}(P_i^n x)| \, d\mu_W = 0; \tag{5}$$

and

$$\int_W \hat{f}(x) \, d\mu_W = \int_{\mathfrak{C}} f(x) \, d\mu_{\mathfrak{C}}. \tag{6}$$

This extended function $\hat{f}(x)$ is uniquely determined by $f(x)$ and the above properties, up to a set outside $\mathfrak{C}$ of μ_W measure zero.

Thus we see that although the measure $\mu_{\mathfrak{C}}$, corresponding to the proposed integral over $\mathfrak{C}$, is not totally additive, various results of integration theory depending on total additivity can be obtained by imbedding $\mathfrak{C}$ in a suitable corona.

This note is dedicated to Carl Siegel in Göttingen on his sixtieth birthday.

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Detailed proofs of the statements made here and additional results may be found in N.Y.U. lecture notes titled, "Integration of Functionals"; and in a future publication.

[1] K. O. Friedrichs, "Quantum Theory of Fields," *Communs. Pure and Appl. Math.*, **4**, 1–64, 1951 (in particular, §12, in which appears the motivation for the present work); I. E. Segal, "A Non-commutative Extension of Abstract Integration," *Ann. Math.*, **57**, 401–417, 1953; "Abstract Probability Spaces and a Theorem of Kolmogoroff," *Am. J. Math.*, **76**, 721–732, 1954.

[2] Another approach to integration over Hilbert space has also been developed by J. Schwartz. In particular, he has obtained a result similar to Theorem A.

[3] The relationship of this theorem to the basic theorem underlying the notion of Wiener integral will be discussed in a later publication.

FACTORIZATION IN THE GROUP ALGEBRA OF THE REAL LINE

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The algebra in question is the set L of all complex Lebesgue integrable functions on the real line, with pointwise addition, and with convolution as multiplication:

$$(g * h)(x) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{\infty} g(x - t)\, h(t)\, dt. \tag{1}$$

The norm

$$\|f\| = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{\infty} |f(x)|\, dx \tag{2}$$

makes L into a Banach algebra. The Fourier transform of a function $f \in L$ will be denoted by $\hat{f}$:

$$\hat{f}(y) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{\infty} f(x)\, e^{-ixy}\, dx. \tag{3}$$

Then $f = g * h$ if and only if $\hat{f}(y) = \hat{g}(y)\, \hat{h}(y)$ for all real y.

In this note it is proved that every member of L is the convolution of two others. Thus, although the algebra L has no unit element, unrestricted factorization is possible. There are no primes in L.

THEOREM. *Suppose that $f \in L$. There exist functions $g \in L$ and $h \in L$ such that*

(*a*) $f = g * h$;

(*b*) *both h and $\hat{h}$ are positive and even;*

(*c*) *g lies in the closed ideal generated by f.*

Proof: For $t > 0$, let K_t be the function whose Fourier transform is

$$\hat{K}_t(y) = \begin{cases} 1 - \dfrac{|y|}{t} & \text{if } |y| < t \\ 0 & \text{if } |y| \geqslant t. \end{cases}$$

Put $\sigma_t = f * K_t$, and $\alpha(t) = \|f - \sigma_t\|$. It is well known that $\alpha(t) \to 0$ as $t \to \infty$.

Choose a sequence $\{t_n\}$ as follows: $t_1 = 0$; for $n \geqslant 2$, $t_n > 2t_{n-1}$ and $\alpha(t) < n^{-2}$ if $t \geqslant t_n$. Construct a function ϕ, concave and with continuous second derivative in $[0, \infty)$, such that $\phi(t_n) = n$. Consideration of the graph of ϕ shows that $t_n \phi'(t_n) < 2$ for $n \geqslant 2$. Hence

$$\begin{aligned} \int_{t_n}^{t_{n+1}} \alpha(t)\, t\, |\phi''(t)|\, dt &< -n^{-2} \int_{t_n}^{t_{n+1}} t\, \phi''(t)\, dt \\ &= n^{-2}\{t_n\, \phi'(t_n) - t_{n+1}\, \phi'(t_{n+1}) + \phi(t_{n+1}) - \phi(t_n)\} \\ &< 3n^{-2}. \end{aligned}$$

Consequently,

$$\int_0^{\infty} \alpha(t)\, t\, |\phi''(t)|\, di < \infty. \tag{4}$$

Now define

$$g(x) = f(x) + \int_0^\infty \{\sigma_t(x) - f(x)\}\, t\, \phi''(t)\, dt. \tag{5}$$

By relation (4) and the Fubini theorem, the integral in equation (5) converges absolutely for almost all x, and $g \in L$. Also,

$$\hat{g}(y) = \hat{f}(y) + \hat{f}(y) \int_0^\infty \{\hat{K}_t(y) - 1\}\, t\, \phi''(t)\, dt.$$

The last integral is an even function of y. For $y > 0$, it is equal to

$$-\int_0^y t\, \phi''(t)\, dt - y \int_y^\infty \phi''(t)\, dt = \phi(y) - \phi(0) = \phi(y) - 1.$$

Thus, if we put $\phi(-t) = \phi(t)$, we have, for all real y,

$$\hat{g}(y) = \hat{f}(y)\, \phi(y). \tag{6}$$

Next, put $\lambda(t) = 1/\phi(t)$, and

$$h(x) = \int_0^\infty K_t(x)\, t\, \lambda''(t)\, dt. \tag{7}$$

Note that λ is convex in $(0, \infty)$, that $\lambda(t) \to 0$ as $t \to \infty$, and that consequently

$$\int_0^\infty t\, \lambda''(t)\, dt = \lambda(0) < \infty.$$

This implies that $h \in L$, and

$$\hat{h}(y) = \int_0^\infty \hat{K}_t(y)\, t\, \lambda''(t)\, dt.$$

For $y > 0$, a calculation similar to the one that led to equation (6) shows that $\hat{h}(y) = \lambda(y)$. By equation (6),

$$\hat{f}(y) = \hat{g}(y)\, \hat{h}(y) \tag{8}$$

for all real y, and part (a) of the theorem is proved.

It is evident from the construction that part (b) holds. To prove part (c), note that the function $\phi\hat{K}_t$ satisfies a Lipschitz condition of order 1 and vanishes outside a bounded interval. Hence $\phi\hat{K}_t = \hat{U}_t$ for some $U_t \in L$, and, by equation (6), $\hat{U}_t\hat{f} = \hat{K}_t\hat{g}$. It follows that $K_t * g$ belongs to the ideal generated by f, for each $t > 0$. As $t \to \infty$, $K_t * g$ tends to g, in the norm of L, so that part c holds. This completes the proof.

It is quite natural to ask now whether every non-negative $f \in L$ is the convolution of two nonnegative members of L. To see that this is not the case, observe that the integral in equation (1) is a lower semicontinuous function of x if g and h are nonnegative, so that f must coincide almost everywhere with some lower semicontinuous function if $f = g * h$. But this is impossible if f is the characteristic function of a compact totally disconnected set of positive measure, for instance.

It would be interesting to determine those functions which are convolutions of nonnegative functions.

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*SPECIFICITY OF THE LONDON-EISENSCHITZ WANG FORCE**

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The London force between macromolecules immersed in a liquid medium has an interesting property which may be of biological significance. For the purpose of formulating the London interaction, one may represent each macromolecule by a set of electric dipole oscillators of specified polarizability, frequency, and orientation. To consider the simplest case, one may study macromolecules of globular form not in direct contact with each other. (They might be separated by Debye-Hückel-Onsager atmospheres made up of molecules from the medium; then the equilibrium distance between the macromolecules would be regulated by concentration changes in the ionic medium.)[1] Such a geometrical arrangement means that the dipole oscillators, which actually are distributed all over a macromolecule, can be replaced by oscillators located at this macromolecule's center. The quadrupole, octupole, etc., terms (which arise when the oscillators are displaced to the molecular center) can be neglected in a crude first approximation.

The following property of the London interaction of macromolecules is to be discussed: Under fairly general conditions, when the London force is very strong and originates from anisotropic oscillators whose polarizabilities, whose frequencies, and whose directions cover diversified distributions for the different types of macromolecules immersed in the same medium, identical macromolecules will associate as near neighbors and adopt a mutual orientation characteristic of anisotropically polarizable molecules. Even though macromolecules or complexes of macromolecules are under discussion, one may, in the following, simply use the word "molecules."

This property can be formulated in the following manner. Consider a system of molecules subject to the following rather general assumptions: (1) the volumes of the different somewhat globular molecules are equal, and only nearest-neighbor interactions, all at the same distance R, are considered; (2) the total number of nearest neighbors of a molecule is the same, on the average, for any arrangement of the system; (3) the interaction is additively made up from pair interactions; (4) the entropy of mixing is ignored.

Let

$$\Delta A_{\mathrm{I\,II}} = (A_{\mathrm{I\,II}})_R - (A_{\mathrm{I\,II}})_\infty$$

represent the free energy of a pair I, II of molecules whose centers are a distance R apart, minus that at infinite separation. Under the four assumptions the difference in free energy between two arrangements of a system of molecules can be shown to be of the form

$$\Delta_4 A_{\mathrm{I\,II}} \equiv \Delta A_{\mathrm{I\,I}} + \Delta A_{\mathrm{II\,II}} - 2\Delta A_{\mathrm{I\,II}},$$

or an integral multiple of it, or a sum of similar terms $\Delta_4 A_{\mathrm{I\,II}}$, $\Delta_4 A_{\mathrm{II\,III}}$, $\Delta_4 A_{\mathrm{III\,I}}$, etc., depending on the number of different types of molecules and the rearrangement under consideration. (This equation defines $\Delta_4 A_{\mathrm{I\,II}}$ as the difference in free energy

between an arrangement I I . . . II II, and an arrangement I II . . . I II, where each row of dots indicates a large separation between the two pairs of near neighbors.) These "rearrangement-free-energy" evaluations can also be interpreted as taking account of "buoyancy": $\Delta_4 A_{\mathrm{I\ II}}$ measures the free-energy gain when two macromolecules I, I, which are immersed in a homogeneous isotropic medium composed of small molecules, become near neighbors; in this case the symbol II stands for any one of the conceptual aggregates of medium molecules into which the medium is parceled out and which are of the same size and shape as the macromolecules I. With this notation, the property under discussion is that $\Delta_4 A_{\mathrm{I\ II}}$ is negative definite.

Consider first the interaction of molecules each represented by a simple isotropic oscillator in the classical limit of oscillator frequencies $\varpi \ll kT/\hbar$:

$$\Delta A_{\mathrm{I\ II}} = -3R^{-6}kT\alpha_{\mathrm{I}}\alpha_{\mathrm{II}}, \tag{1}$$

$$\Delta_4 A_{\mathrm{I\ II}} = -3R^{-6}kT(\alpha_{\mathrm{I}} - \alpha_{\mathrm{II}})^2 \leq 0. \tag{2}$$

J. H. de Boer and H. C. Hamaker[2] have found a corresponding and more interesting inequality from the London formula[3] which refers to the quantum limit $\varpi \gg kT/\hbar$; ϖ_{I} and ϖ_{II} are the frequencies of I and II when separated (at $R = \infty$),

$$\Delta A_{\mathrm{I\ II}} = -(3/2)R^{-6}\alpha_{\mathrm{I}}\alpha_{\mathrm{II}}\hbar\varpi_{\mathrm{I}}\varpi_{\mathrm{II}} \Big/ (\varpi_{\mathrm{I}} + \varpi_{\mathrm{II}}) \tag{3}$$

$$\Delta_4 A_{\mathrm{I\ II}} = -3/4R^{-6}\hbar\,\frac{(\alpha_{\mathrm{I}}\varpi_{\mathrm{I}} - \alpha_{\mathrm{II}}\varpi_{\mathrm{II}})^2 + \varpi_{\mathrm{I}}\varpi_{\mathrm{II}}(\alpha_{\mathrm{I}} - \alpha_{\mathrm{II}})^2}{\varpi_{\mathrm{I}} + \varpi_{\mathrm{II}}} \leq 0. \tag{4}$$

If each molecule is adequately represented by a *set* of oscillators, no essential change occurs in equations (1) and (2). The sum of the polarizabilities of all the oscillators in molecule I is inserted in place of the single oscillator polarizability α_{I} in equation (2), and similarly with the polarizabilities of molecule II.[4] This means that there is an inequality just like equation (2), depending on a single quantity, i.e., the difference of the total polarizabilities of the two molecules. The same holds good for equations (3) and (4), if all the oscillators have one and the same frequency. (All this becomes evident from eq. [10] below.) Conversely, equation (4) and its multi-oscillator generalization become of general interest if the oscillators cover a diversified range of frequencies as well as of polarizabilities.

If one represents actual macromolecules by oscillator sets, these sets will usually show such a wide distribution of frequencies that neither the classical nor the quantum limit results can serve as a basis for the discussion of specificity.

Previous to knowing the pioneer work of Hamaker and de Boer, we proceeded in the following manner, which should serve the purpose of defining and estimating this kind of specificity. This procedure covers the many-oscillator case and covers the entire range of frequencies.

The partition function Z of a pair of molecules can be expressed in terms of the normal mode frequencies ω_l of the molecule *pair;* ω_l includes the effect of the intermolecular interaction of the oscillators. This leads to the free energy

$$A_{\mathrm{I\ II}} = -kT \ln Z = kT \sum_{l=1}^{N_{\mathrm{I}} + N_{\mathrm{II}}} \ln\left[2 \sinh\left(\frac{\hbar\omega_l}{2kT}\right)\right] \tag{5}$$

$$= kT \sum_l \left\{ {}^1/_2 \ln \frac{\hbar^2\omega_l^2}{4k^2T^2} + \sum_{s=1}^{\infty} \ln\left(1 + \frac{\hbar^2\omega_l^2}{4k^2T^2}\frac{1}{s^2\pi^2}\right) + \ln 2 \right\}. \quad (6)$$

One can replace this sum over the normal modes by the trace of a function of the diagonalized potential-energy matrix (with eigenvalues ${}^1/_2\omega_l^2$) and take advantage of the fact that this trace is invariant, i.e., the same as the trace of the potential-energy matrix which has the intermolecular interaction entries

$$U_{lj} \propto \epsilon_l m_l^{-1/2} \epsilon_j m_j^{-1/2} R^{-3}(u_{lx}u_{jx} + u_{ly}u_{jy} - 2u_{lz}u_{jz})$$

still present in off-diagonal locations; ϵ_l, m_l, u_{lx}, u_{ly}, u_{lz} are the effective charge, mass, and direction cosines of the lth oscillator of the isolated molecule I, and the subscript j refers correspondingly to molecule II. The form (6) readily permits expansion of $\Delta A_{\mathrm{I\,II}}$ in powers of U. If U can be written as a matrix product of one factor referring to molecule I only, and another to molecule II (and this is certainly possible for the dipolar part of the polarizability interaction), then, after some matrix calculations,[5] one obtains

$$\Delta A_{\mathrm{I\,II}} = -{}^1/_2 kT \text{ trace } \sum_{s=-\infty}^{+\infty} W_{s\mathrm{I}} W_{s\mathrm{II}}, \quad (7)$$

where

$$W_{s\mathrm{I}} = -R^{-3} \sum_{l=1}^{N_\mathrm{I}} \frac{\alpha_l}{1 + (4\pi^2k^2T^2/\hbar^2\varpi_l^2)s^2} \begin{pmatrix} u_{lx}^2, & u_{lx}u_{ly}, & \sqrt{2}u_{lx}u_{lz} \\ u_{ly}u_{lx}, & u_{ly}^2, & \sqrt{2}u_{ly}u_{lz} \\ \sqrt{2}u_{lz}u_{lx}, & \sqrt{2}u_{lz}u_{ly}, & 2u_{lz}^2 \end{pmatrix}. \quad (8)$$

Here $\alpha_l = \epsilon_l^2/m_l\varpi_l^2$ is the static polarizability and ϖ_l the frequency of the lth oscillator of the isolated molecule I. An expression corresponding to equation (8) holds for II, the proviso being that if the oscillator orientations of molecule I are referred to axes x_I, y_I, z_I, the oscillators of molecule II should be referred to axes x_II, y_II, z_II, where z_I and z_II point in the same direction, along the line connecting the molecule centers I and II, and where x_I and x_II are antiparallel and so are y_I and y_II. (One verifies easily that expressions [7] and [8] yield, in the classical and quantum limits, formulas [1] and [3], respectively, or their anisotropic generalizations.) A pair of molecules is called an *identical* pair if the molecules themselves are identical and if their orientations correspond to a 180° screw translation along the z axis. Mirror-image pairs do not associate, as their permanent dipole moments prefer orientations different from those required by the oscillators. With these notations, the rearrangement free energy becomes

$$\Delta_4 A_{\mathrm{I\,II}} = -{}^1/_2 kT \sum_{s=-\infty}^{+\infty} \sum_{\mu\nu=1}^{3} \{(W_{s\mathrm{I}} - W_{s\mathrm{II}})_{\mu\nu}\}^2 \leq 0. \quad (9)$$

For a one-dimensional set of oscillators this becomes

$$\Delta_4 A_{\mathrm{I\,II}} = -{}^1/_2 kT \sum_{s=-\infty}^{+\infty} 4R^{-6} \left[\sum_{l=1}^{N_\mathrm{I}} \frac{\alpha_l}{1 + (2\pi kT/\hbar\varpi_l)^2 s^2} - \sum_{l=N_\mathrm{I}+1}^{N_\mathrm{I}+N_\mathrm{II}} \frac{\alpha_l}{1 + (2\pi kT/\hbar\varpi_l)^2 s^2} \right]^2 \leq 0, \quad (10)$$

so that this is a sum over s, to which, in the classical limit, only $s = 0$ contributes; the important contributions go up to about $|s| = 100$ if near-ultraviolet oscillators have the strongest polarizabilities. Relation (10) is not additively composed of contributions from the oscillators $l = 1, 2, \ldots$.

Inequality (10) is made up of a sum of negative-definite terms; it is therefore equivalent to several inequalities which, of course, are not all independent. Should there be, among the different kinds of molecules, only two different narrow frequency regions with appreciable polarizabilities α_l (e.g., one region in ultraviolet, the other in infrared [Fig. 1]), then we would have only two effectively independent inequalities. Going from relation (10) to the three-dimensional case, relation (9), the number of independent inequalities increases. This occurrence of several inequalities presents an interesting specificity.

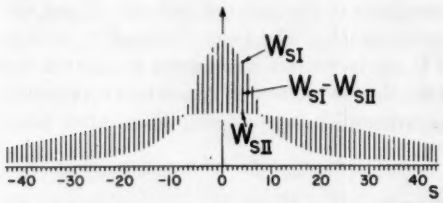

FIG. 1.—Illustration of equations (9) and (10) for one dimensional oscillators in the simplest case when, in molecule I, $\hbar\bar{\omega}_l/2\pi kT = 7$ (infrared) for all $l = 1$ to N_I, and, in molecule II, $\hbar\bar{\omega}_l/2\pi kT = 37$ (ultraviolet) for all $l = N_I + 1$ to $N_I + N_{II}$.

So far, this note has discussed particular rearrangement free energies arising from a given pair of molecule types I and II. The concept "specific interaction" may be attached to the capacity of discrimination which a particular molecule type I exhibits in its interaction with other molecule types II taken at random out of a *manifold* of molecule types. The degree of specificity thus may be defined as the measure of the subset of types II discriminated against when confronted with type I, divided by the measure of the total set of all types II in the manifold. The interesting feature of the many-parametric distribution of W_s, i.e., of the several independent inequalities, is that, even though the average of the various rearrangement free energies $\Delta_4 A_{I\ II}$ may be quite moderate, the degree of specificity so defined can be fairly high, close to unity, simply by virtue of the many-dimensionality of inequality (9) or inequality (10).

Even in the absence of adequate experimental data concerning the polarizabilities and the intermolecular distances R, a brief remark about the order of magnitude of the effects may be appropriate. London–van der Waals forces are indeed weak in general. If one measures the interaction energy by comparison with a fixed quantity kT, defining as "range" that distance R at which this energy is equal to $-kT$, and if one measures the total polarizability of a molecule in terms of its volume, then the range will have to be measured in terms of the molecular diameter, because $(\text{volume})^2 \times R^{-6}$ depends only on the ratio $(\text{molecular diameter}/R)$. This means that for macromolecules the London interaction may reach farther than ordinary chemical bonds do. London specificity effects can, however, manifest themselves only if the molecules have very strong polarizabilities and if these polarizabilities are distributed over a very wide frequency range from the ultraviolet down, and with diversified oscillator orientations, these distributions being quite different for the various molecule types. The crude overall distribution is all what matters, the finer details are quite irrelevant; this is evident from Figure 1.

The biological significance of this property of the London force may be exemplified in the problem of synapsis of homologous chromosome sections during meiosis. That there is accurate recognition of corresponding parts of a chromosome pair (which have to be considered as approximately identical rather than complementary to each other) is evidenced strikingly in the phenomenon of inverted synapsis. The mechanism which brings homologous chromosome sections together and lets them go apart again at a later time might perhaps be regulated by ionic concentration changes in the medium.

Still more important is the specificity of the London force for an understanding of self-duplication. It does not seem likely that the genes unfold or rip into two halves in the process of self-duplication, for two reasons: (1) The enormous stability of a gene capable of surviving millions of duplication processes, unharmed, appears to be a phenomenon which is rather incompatible with a duplication mechanism which does not leave the gene entirely intact, because an unfolded structure (or, even more so, a Watson-Crick double helix if ripped apart) would be liable to breakages or other changes. (2) The astonishing accuracy of the duplication process is equally difficult to comprehend if the structure representing the gene is not kept intact. The opening up of such a structure would permit "alien" groups of atoms to become attached and thereby change the gene.

One may assume that molecules out of which the genes can be assembled are readily available among many other molecules in the medium surrounding a gene and that Brownian motion provides for a reshuffling of those molecules. The specificity of the London force, if strong enough, will then cause the retention of medium molecules, which happen to be identical with the constituent molecules of the gene, respectively, in the neighborhood of the gene molecules. This will considerably facilitate the assembly process.[6]

With regard to the properties of the London force, molecules are identical if they have the same distribution of polarizabilities. Structural identity is a sufficient but not a necessary condition for the "identity" on which London-force specificity depends. Correspondingly, London specificity may play a role in a wider group of biological specificity phenomena, such as enzyme specificity or antigen-antibody specificity.

It is evident that the manifestation of biological specificity is due to several quite distinct phenomena. Specificity based on complementarity is the best known among them and has reached the stage of quantitative evaluation.[7] A detailed account of this theory and a more comprehensive list of references will be given elsewhere.[5]

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[1] Cf. J. Th. G. Overbeek's review of Hamaker's work in H. R. Kruyt, *Colloid Science* (New York: Elsevier Publishers, 1952), **1**, 276, 277.

[2] H. C. Hamaker, *Physica*, **4**, 1058, 1937; J. H. de Boer, *Trans. Faraday Soc.*, **32**, 118, 1936.

[3] F. London, *Discussions Faraday Soc.*, September, 1936, pp. 8–26; *Z. physik. Chem., B*, **11**, 222, 1930.

[4] The Kirkwood-Shumaker dipole moment fluctuations (mobile proton contributions to the polarizability) contribute substantially to this classical limit case, these PROCEEDINGS **38**, 855 and 863, 1952.

[5] J. M. Yos, W. L. Bade, and H. Jehle, in *Symposium on Molecular Structure and Biological Specificity*, ed. L. Pauling and H. Itano (American Institute of Biological Sciences, 1957).

[6] H. J. Muller, *Am. Naturalist*, **56**, 32, 1922; *Sci. Monthly*, **44**, 210, 1937; *Cold Spring Harbor Symposia Quant. Biol.*, **9**, 290, 1941; *Proc. Roy. Soc. London, B*, **134**, 1, 1947; *Genetics in the 20th Century*, ed. L. C. Dunn (New York: Macmillan Co., 1951), p. 77.

[7] Linus Pauling, *J. Am. Chem. Soc.*, **62**, 2643, 1940; *Nature*, **161**, 707, 1948; *Stoll Festschrift* (Basel, 1957); Felix Haurowitz, *Chemistry and Biology of Proteins* (New York: Academic Press, 1950), D. H. Campbell, *Principles of Immunology* (New York: McGraw-Hill, 1957).

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*THE AUDITORY SENSITIVITY OF THE ATLANTIC GRASSHOPPER**

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A number of investigators have made use of the electrophysiological method for the study of auditory sensitivity in insects. The method consists of the observation, during stimulation by sounds, of impulses produced in the nerve supplying the tympanal organ or, in some instances, of impulses in the thoracic ganglion to which this nerve runs. Wever and Bray[1] in their introduction of this method in 1933 reported results on two species of katydids, *Amblycorypha oblongifolia* and *Pterophylla camellifolia*, and one species of cricket, *Gryllus assimilis*. Soon thereafter, Wever[2] obtained curves of threshold sensitivity in the sulfur-winged grasshopper, *Arphia sulphurea*. This method has since been used by Pumphrey and Rawdon-Smith[3] on the grasshopper *Locusta migratoria migratorioides;* by Autrum[4] on the katydids *L. viridissima*, *Decticus verrucivorus*, and *L. cantans;* by Benedetti[5, 6] on several species of Orthoptera, including *Sphyngonotus coerulans*, *Anacridium aegyptum*, and *L. viridissima;* and most recently by Tischner[7] on the mosquito *Anopheles subpictus*. In the present study the method has been used in measurements of threshold sensitivity in the Atlantic grasshopper, *Paroxya atlantica* (Scudder).

The insect was first prepared by removing the head, legs, wings, and the posterior portion of the abdomen, in order to reduce its mobility. The body portion was mounted on a pedestal of modeling clay, and an opening was made on the left side to give an exposure of the right tympanal nerve. In this exposure the left tympanal organ was removed, along with a part of the body wall. Also removed was a large mass of eggs or sperm, with which the insects were laden at this season (early November). Despite the extensive dissection, the usual respiratory move-

ments were unimpaired, and the tissues remained in good condition as long as they were protected against drying.

The preparation was then located opposite a sound tube, with the tympanic membrane at a standard distance from the end of the tube. Sound intensities were measured by placing the tip of a calibrated probe tube connected to a condenser microphone at the same position as the tympanic membrane. An active electrode, consisting of a fine cotton thread soaked with saline solution, was placed on the tympanal nerve, and another electrode in the form of a steel needle was located in inactive tissue near by. The nerve currents aroused by acoustic stimulation were amplified and conducted to a telephone receiver, through which they became audible as sound.

Pure tones over a range from 100 to 15,000 cycles per second were used as stimuli and were varied in intensity in steps of 2–5 db to determine a threshold. The threshold was definite: as the intensity was raised from a low level, the discharge appeared suddenly at an easily perceptible magnitude. At the threshold the impulses were relatively few, but as the intensity was raised further, they increased in number until a maximum was reached a few decibels above threshold.

At the low frequencies, up to 200 cycles, there was a fair degree of synchronism between the impulses and the stimulus waves. From 250 to 400 cycles this synchronism diminished, and beyond 400 cycles the discharge seemed wholly asynchronous.

Detailed threshold measurements were carried out on six animals, and in Figure 1 we show the median values obtained. For comparison we present in the same

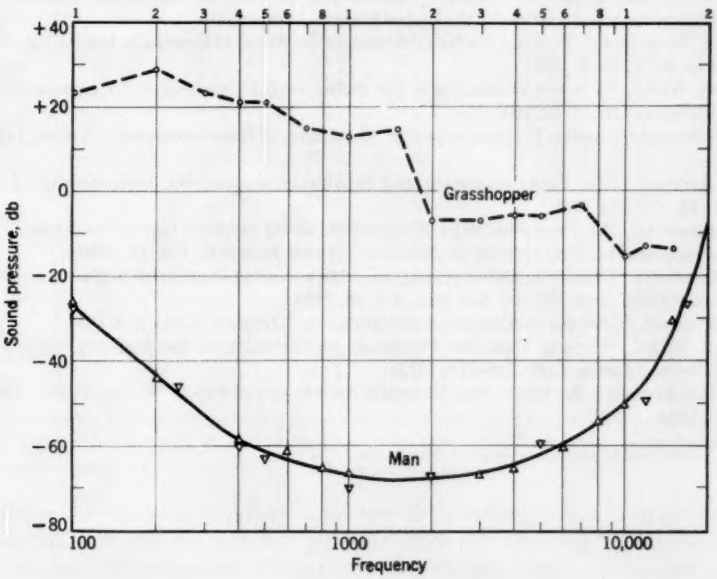

FIG. 1.—The threshold sensitivity of the Atlantic grasshopper in comparison with that of man. Sound pressure is shown in decibels relative to 1 dyne per square centimeter.

graph the average human threshold curve for uniaural listening with a telephone receiver as determined in two experiments, one by Wegel[8] (*upright triangles*) and one by Munson[9] (*inverted triangles*).

As the curve shows, the tympanal organ of the grasshopper is relatively insensitive at the low frequencies but improves as the frequency is raised. The improvement occurs at a fairly regular rate of 6–7 db per octave, which suggests that within this range the vibratory response of the tympanal organ is largely determined by its stiffness. At the highest frequencies shown, the function still represents poorer sensitivity than the human curve, but from its trend it seems likely that at still higher frequencies it would cross that curve. Impulses were obtained for tones beyond 15,000 cycles, but because the microphone calibration extended only to this frequency, it was not possible to make further quantitative measurements.

These results are similar in form to those previously obtained on other species of grasshoppers. The sulfur-winged grasshopper, *Arphia sulphurea*, studied by Wever, showed about 10 db less sensitivity in the low frequencies (300–1,000 cycles) and about 6 db greater sensitivity in the high frequencies (6,000–10,000 cycles). The migratory grasshopper *L. migratoria migratorioides*, studied by Pumphrey and Rawdon-Smith, showed about the same sensitivity as *Paroxya atlantica* in the low frequencies (400–1,000 cycles), but considerably greater sensitivity in the high frequencies. These differences may be due in part to differences of technique, but it is also possible that they represent true species variations.

* This research was supported in part by a contract with the Office of Naval Research (Contract N6-onr-270-3) and in part by Higgins Funds allotted to Princeton University. Permission is granted for reproduction and use by the United States Government.

[1] E. G. Wever and C. W. Bray, "A New Method for the Study of Hearing in Insects," *J. Cellular Comp. Physiol.*, **4**, 79–93, 1933.

[2] E. G. Wever, "A Study of Hearing in the Sulfur-winged Grasshopper (*Arphia sulphurea*)," *J. Comp. Psychol.*, **20**, 17–20, 1935.

[3] R. J. Pumphrey and A. F. Rawdon-Smith, "Sensitivity of Insects to Sound," *Nature*, **137**, 990, 1936.

[4] H. Autrum, "Über Lautäusserungen und Schallwahrnehmung bei Arthropoden," *Z. vergl. Physiol.*, **28**, 326–352, 1940.

[5] E. Benedetti, "La Percezione degli ultrasuoni in alcuni ortotteri rilevata mediante la registrazione oscillografica delle correnti bioelettriche," *Ateneo parmense*, Vol. **22**, 1950.

[6] E. Benedetti, "Comportamento e propagazione dei potenziali di azione di origine neuroacustica in alcuni ortotteri," *Boll. Soc. ital. biol. sper.*, Vol. **26**, 1950.

[7] H. Tischner, "Über den Gehörsinn von Steckmücken," *Acustica*, **3**, 335–343, 1953.

[8] R. L. Wegel, "Physical Data and Physiology of Excitation of the Auditory Nerve," *Ann. Otol. Rhinol. and Laryngol.*, **41**, 740–779, 1932.

[9] L. J. Sivian and S. D. White, "On Minimum Audible Sound Fields," *J. Acoust. Soc. Amer.*, **4**, 288–321, 1933.

*SOME EFFECTS OF ALTERNATION RATE IN A TIME-CORRELATED REINFORCEMENT CONTINGENCY**

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In an earlier paper[1] describing a system of temporal parameters for classifying reinforcement schedules, two experiments were cited as in progress, and sample response curves for each were shown. We are now in a position to give the final function, as well as some additional data, for the first of those experiments,[2] namely, that in which $t^D/(t^D + t^\Delta)$ was constant at 0.05, while cycle length, $t^D + t^\Delta$, varied through values of 30.0, 15.0, 7.5, 3.75, 1.88, and 0.94 seconds, in that order. Details of the experimental procedure are given in the earlier paper.

Table 1 contains the individual data of the four birds employed in the experiment. An "uncorrected rate" entry represents the mean response-per-minute

Table 1

Means (for Six Stability Criterion Days) of Uncorrected and Corrected Response Rates per Minute, and of Responses per Reinforcement ($R's/S^R$), for Each Experimental Animal for Each Cycle Length, $t^D + t^{\Delta*}$

	Cycle Length (Secs.)					
	30.0	15.0	7.5	3.75	1.88	0.94
Bird No. 7:						
$Rate_{uncorr.}$	30.7	50.1	59.3	79.2	110.9	108.2
$Rate_{corr.}$	34.1	62.4	91.8	104.7	160.1	141.4
$R's/S^R$	15.7	12.7	8.4	16.3	18.1	23.1
Days to criterion	19	15	13	14	17	15
Bird No. 8:						
$Rate_{uncorr.}$	21.5 [101.2]	36.0	68.7 [116.4]	139.5	126.9	163.7
$Rate_{corr.}$	23.3 [112.2]	40.3	90.9 [176.6]	209.5	201.4	283.0
$R's/S^R$	14.1 [51.4]	16.7	14.1 [17.1]	20.9	17.1	19.4
Days to criterion	17	13	13	15	17	14
Bird No. 9:						
$Rate_{uncorr.}$	23.9	40.5	71.5	89.0	91.7	95.5
$Rate_{corr.}$	25.9	49.3	104.2	121.2	120.3	138.6
$R's/S^R$	15.5	11.4	11.4	16.8	19.3	15.3
Days to criterion	15	13	13	13	14	14
Bird No. 4:						
$Rate_{uncorr.}$	29.1	44.2	67.1	71.3	69.2	...
$Rate_{corr.}$	32.2	53.3	96.7	90.0	83.5	...
$R's/S^R$	14.9	13.0	11.0	17.1	20.1	...
Days to criterion	21	13	14	21	13	...
Mean (bird Nos. 7, 8, 9):						
$Rate_{uncorr.}$	25.7	42.2	66.5	102.6	109.8	122.5
$Rate_{corr.}$	27.8	50.7	95.6	145.1	160.6	187.7
$R's/S^R$	15.1	13.6	11.3	18.0	18.2	19.3

* Also shown are the numbers of experimental sessions required by each animal to meet the stability criterion at each cycle length. The value of $t^D/(t^D + t^\Delta)$ is constant throughout at 0.05. The bracketed entries for bird No. 8 are the values obtained on return to cycle lengths of 7.5 and 30.0 seconds after completion of the function. The means at the bottom omit the values of bird No. 4. (Further details in text.)

rate obtained by summing responses in the last 15-minute portion of each 20-minute daily session over the six stability criterion days and dividing by 90 minutes; "corrected rate" takes account of the time spent by each bird in eating and is obtained by taking the same sum of responses as before, but subtracting from 90 minutes the total amount of time during which the food hopper was presented

(number of reinforcements multiplied by 3 seconds); "responses/reinforcement" ($R's/S^R$) presents the same sum of responses divided by total reinforcements procured during the 90-minute total time of the six criterion days; "days to criterion" represents the total number of daily sessions, including the six criterion days, required for each bird to meet the response stability criterion and to qualify for shift to the next cycle length.[3] The means given at the bottom of Table 1 do not include the data of bird No. 4. This animal began to show increasing variability in day-to-day response rate after reaching the cycle length of 3.75 seconds, on which 21 days were taken to meet the stability criterion; at the 1.88-second cycle length her weight began to fall so that she needed supplemental feeding after each day's session, and her daily response variability was clearly unusual, although the stability criterion was met by chance on the thirteenth day in the course of widely fluctuating daily response totals. After being shifted to the 0.94-second cycle length, her weight soon fell markedly below 80 per cent of her weight under ad lib. feeding despite supplemental rations; thereafter, for no assignable reason, ingestion of her supplemental rations diminished over a period of several weeks and finally ceased, with eventual death from inanition. Although bird No. 4's data do not seem suspect for the cycle lengths of 30.0, 15.0, and 7.5 seconds, all her data are excluded from the group means. Figure 1 (*main section*) plots the mean uncorrected

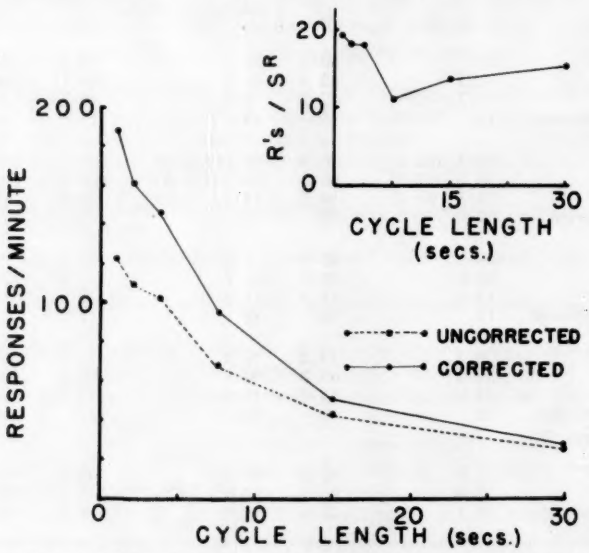

FIG. 1.—Means of uncorrected and corrected response rates per minute as a function of cycle length ($t^D + t^\Delta$), the plotted values being those at the bottom of Table 1. The inset graph is that of mean responses per reinforcement ($R's/S^R$) as a function of the same cycle lengths.

and corrected rates given at the bottom of Table 1. The functions approach an asymptote at the longer cycle lengths, the value of the asymptote perhaps being, at least in part, an effect of the experimental conditions used to inaugurate respond-

ing in this case, 14 days of training, with $t^D/(t^D + t^\Delta) = 1.00$, $t^D + t^\Delta = 30.0$ seconds.[4] This lower asymptote is the same for both corrected and uncorrected rate functions, since the correction factor becomes negligible as frequency of reinforcement diminishes. Presumably, there is also an asymptote to the functions for response rates below the 0.94-second cycle, since a rate ceiling must exist for each bird, and indications of this are seen in the individual data of birds Nos. 7 and 9.

The bracketed entries for bird No. 8 in Table 1, at cycle lengths of 7.5 and 30.0 seconds, were obtained after this animal's complete function had been secured, since it was deemed of interest to see whether response rates could be recovered on return to earlier values of the independent variable. It will be recalled that, in the experiment proper, each bird was exposed to the cycle lengths in order of decreasing length. Bird No. 8 (for which some sample cumulative response curves were shown in the earlier paper) was selected for this attempt at recovery of response rate because her rate at the 0.94-second cycle was the highest in the group and thus seemed to afford the most stringent test of recoverability at hand. After completing her function at the 0.94-second cycle, the bird's daily sessions were immediately returned to the 7.5-second cycle and were continued there for 34 days, after which a shift was again made to the 30.0-second cycle for another 34 days; experimental procedure and conditions were the same as they had been during the original work at these cycle lengths. At both the 7.5- and 30.0-second cycles the bird met the usual 6-day stability criterion several times before the thirty-fourth day, as well as on day 34, but was continued past the criterion to further assure the result.

The recovered rates of bird No. 8 at the 7.5- and 30.0-second cycles indicate that something akin to a behavorial "elastic limit," as it were, had been passed with the emergence of the higher response rates at shorter cycle lengths,[5] and the effect of this appears as well in the responses-per-reinforcement data. The recovered value for the 30.0-second cycle is probably affected somewhat by the fact that the 7.5-second cycle recovery sessions preceded it. With only two recovered values,[6] the form of the new function, moving systematically from 0.94 second back through the longer cycles to 30.0 seconds and beyond, is not clear, but several possibilities are conceivable when one keeps in mind the fact that indefinite lengthening of the $t^D + t^\Delta$ cycle must eventually produce extinction.

The failure wholly to recover rates is explicable on analytic grounds as well as from actual observation of bird No. 8's behavior. Once a high response rate is established under short cycles, return to a longer cycle permits the reinforcement contingency to be realized in two ways: first, rapid responding still gets reinforced, though "superstitiously,"[7] if continued long enough; and, second, temporally discriminated responding, involving pauses after each reinforcement and requiring fewer responses per reinforcement, also may be reinforced. The first of these keeps the high rates generated by the short cycles from subsiding. This is borne out by observations of the actual behavior of bird No. 8: first, within a daily session, at both of the retested cycle lengths, and even after a full month's training, responding between reinforcements fluctuated between low temporally discriminated rates and high ratio-type rates; second, beyond the afore-mentioned local fluctuations, a daily session often showed a slower phasic change in response rate;[8] third, on occasions when a reinforcement was "missed" by low temporally discriminated responding, there would suddenly emerge a high rate which was generally

sustained until the next reinforcement was procured and which sometimes persevered for a while thereafter; and, fourth, the immediate result of the shift from the 7.5-second cycle to the 30.0-second cycle was a jump in rate almost back to the earlier value at the 0.94-second cycle and lasting for the first five sessions at the 30.0-second cycle, pointing to an effect of the new lowered frequency of reinforcement analogous to that arising from both shorter cycle lengths and from the aforementioned "missing" of reinforcements. Behavioral functions like the present ones, obtained from single organisms, each serving as its own control and brought to response equilibrium or "steady state" under systematically changing and successive values of an experimental independent variable, are not, of course, invalidated by any such "inelasticity." The phenomenon does impose, however, an added condition upon the interpretation of such functions; further, it carries several implications for the design of behavioral experiments, for example, the desirability in cases like the present of avoiding the randomly ordered use of independent variable values, in favor of such a systematic exploration of the continuum as would not contaminate the primary effect of the variable with any irreversibility. Aside from such considerations, however, any "inelasticity" demonstrable in a behavioral function is as important in its own right as the function itself.

Figure 1 (*inset*) plots the mean values of responses/reinforcement given at the bottom of Table 1. As seen, the value at the shortest cycle length approximates the 20 responses per reinforcement expected on the ground that, with a temporal discrimination nearing unattainability, the organism becomes a "random responder" (or "probability responder"), with the probability of reinforcement for any response being defined by $t^D/(t^D + t^\Delta) = 0.05$. The minimum in the function, seemingly genuine enough, since it is reflected in the data of the individual birds, is not yet understood.

Figure 2 shows cumulative response curves for six extinction sessions given bird No. 7 following her completion of work on the 0.94-second cycle. These extinction sessions occurred on six successive days under exactly the same procedure as during the regular experimental days, save that no reinforcements were allowed; the omission of reinforcements necessitated supplemental food rations after each day's session to keep the bird at the 80 per cent weight level. The curves are for the whole of the 20-minute sessions rather than only the last 15 minutes. In view of the proposed system of temporal parameters for classifying reinforcement schedules, it was thought to be of interest to see what properties extinction curves would exhibit after the particular history and sequence of training given in the present experiment, where, it was argued, response characteristics shift from "interval" to "ratio" ones as cycle length shortens. Not unexpectedly, the extinction curves show an admixture of properties, some typical of extinction responding after "interval" reinforcement schedules and others typical of curves after a "ratio" reinforcement history.

Summary.—In an earlier paper a system of temporal parameters was proposed for integrating "interval" and "ratio" reinforcement schedules within a single framework. Final functions are presented for an experiment, employing pigeons and a pecking response, in which $t^D/(t^D + t^\Delta) = 0.05$, while $t^D + t^\Delta$ varies through the values of 30.0, 15.0, 7.5, 3.75, 1.88, and 0.94 seconds, in that order. Extinction responding following completion of this function exhibits an admixture of features

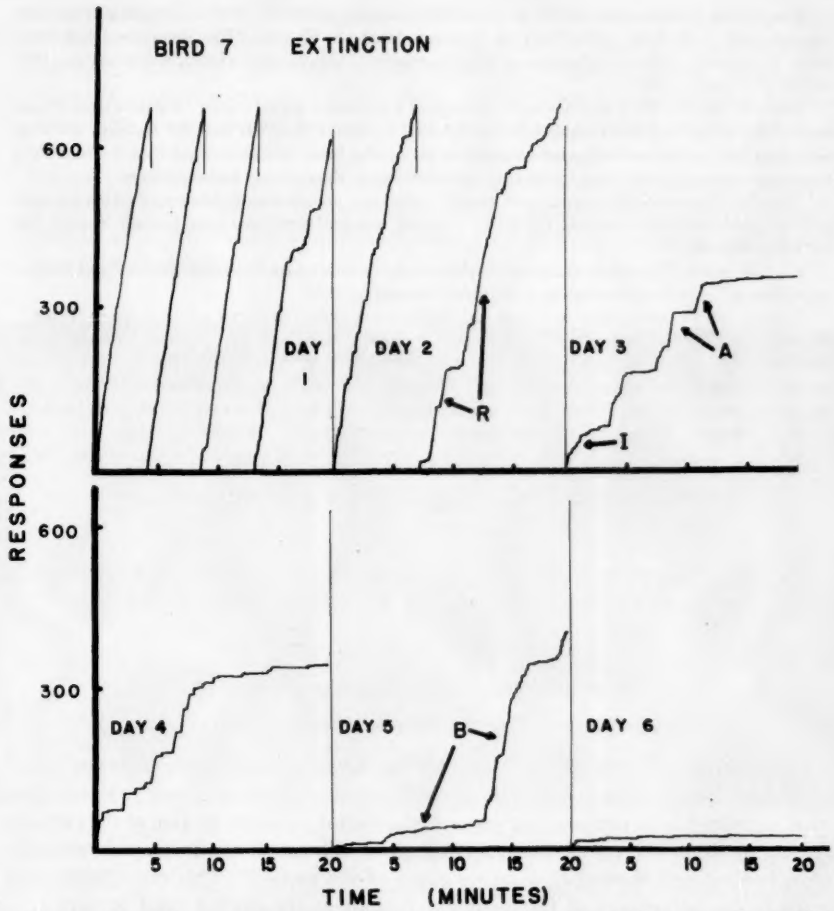

FIG. 2.—Cumulative response curves for six consecutive sessions of extinction responding by bird No. 7, taken immediately following completion of this animal's work in the main experiment. Responding on day 1 is largely of the "ratio" type, similar to the bird's performance on the shorter cycle lengths. Thereafter, the curves show properties characteristic of both long and short cycle lengths, i.e., an admixture of features usually seen during extinction after "interval" and "ratio" reinforcement. Typical of post-"ratio" extinction are the response "bursts" and "breaks," as at *R;* typical post-"interval" extinction is seen at *I;* "bursts" of responding may occur at different rates appropriate to the several cycle lengths incorporated in the animal's reinforcement history, as shown at *A;* finally, an example of post-"interval" responding giving way to post-"ratio" responding is found at *B*.

typically produced by "interval" and "ratio" reinforcement histories. Supplementary data bearing on response rate recoverability are presented and discussed, and some experimental implications of these data are considered.

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[1] W. N. Schoenfeld, W. W. Cumming, and E. Hearst, "On the Classification of Reinforcement Schedules," these PROCEEDINGS, **42**, 463–570, 1956.

[2] The second experiment (with $t^D + t^\Delta = 30.0$ seconds and $t^D/(t^D + t^\Delta)$ decreasing from 1.00 through 0.35, 0.05, 0.03, 0.02, 0.013) is also completed (E. Hearst, "The Behavioral Effects of Some Temporally Defined Schedules of Reinforcement," unpublished Doctor's dissertation, Columbia University, 1956).

[3] Days on which a bird was not used because of a deviation greater than 15 grams from 80 per cent ad lib. weight are not included in this figure. It should be noted that the stability criterion both here and in the earlier paper was computed on the basis of uncorrected rate. Probably a better course would have been to use the corrected rate in the stability computations.

[4] Data for the initial 14 training days are not presented, nor are comparisons made with the later findings presented here, because the initial training was not continued long enough to meet our stability criterion.

[5] Cf. J. J. Boren, "Response Rate and Resistance to Extinction as Functions of the Fixed Ratio," unpublished Doctor's dissertation, Columbia University, 1953.

[6] During the 34 days taken to obtain the 30.0-second cycle recovered value given in Table 1, response rate reached a lower value (on day 26, with uncorrected rate = 66.6 responses/min, corrected rate = 73.5 responses/min) that just missed meeting the criterion, the stability computation yielding 0.0507 rather than <0.05. Thereafter, however, rate increased until the thirty-fourth day, when the stability criterion was again reached at the higher rate shown in the table.

[7] B. F. Skinner, "Superstition in the Pigeon," *J. Exptl. Psychol.*, **38**, 168–172, 1948.

[8] Cf. B. F. Skinner, *The Behavior of Organisms* (New York: D. Appleton-Century Co., 1938), pp. 123–126.

TRANSPLANTATIONS OF THE EMBRYONIC CHICK FOREBRAIN REGION BEFORE ESTABLISHMENT OF CIRCULATION

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In exploring the technique involving routine decapitation of chick embryos before the establishment of circulation, *ca.* 33–36 hours of incubation, it was observed that what appeared to be primary healing took place after complete section of the nervous system in the mid-level of the mesencephalon. The embryos developed normally after healing and showed little or no effect of the section. This observation was made in the Laboratory of Development Biology at the Rockefeller Institute.

It seemed that if the chick embryo could successfully withstand this operation, it might be possible to detach completely the forebrain region of the head and then replace it in its original location as an autograft. After success had been achieved in a significant number of autografts, the technique was extended to include cross transplantations, homografts, of forebrain regions from the same breed of chicks and also from breeds different from the hosts. This report is based on 116 grafting operations, of which 40 cases were significantly successful.

The technique used in this work is quite simple, but the results showed a wide range of variation. A pair of watchmaker's forceps, adapted to the type of operation, was used for cutting through the embryonic mesencephalon. In detaching the forebrain region, care must be taken not to injure or displace the proamnion, which permits the yolk to flood the upper surface of the membrane, often causing fatality. Failure of the proamnion to fold and to undercut the embryo causes the viscera, sometimes including the heart, to remain uncovered to the last day of

incubation. If the forebrain region is removed with forceps, there is little danger of displacing the proamnion, but it is much easier to employ a suction pipette. Capillary attraction exercised by the inmoving extra-embryonic fluid provides adequate negative pressure. The tissue intended for transplantation is placed directly on the previously prepared graft bed in the host without immersion in salt solutions. This eliminates a step which, judging from observations on behavior of tissues in vitro, may be deleterious. The tissue is removed from the pipette by gentle positive pressure. It is desirable that the whole operation be completed as quickly as possible to prevent drying of the graft or the initiation of healing in the wound of the host.

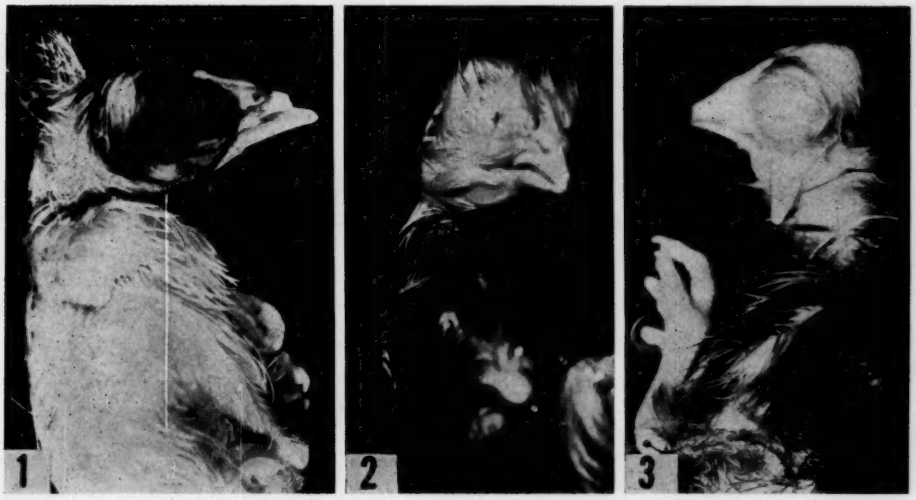

FIG. 1.—20-day-old White Leghorn embryo with grafted Barred Rock Black embryonic forebrain region Host's lower jaw longer and out of position in relation to the upper jaw that has developed from the graft. Left eye underdeveloped or, possibly, absent.

FIG. 2.—22-day-old Barred Rock Black embryo with grafted Rhode Island Red embryonic forebrain region. Mandible slightly out of position in relation to the maxilla. Black and red feathers sharply demarcated. This embryo attempted to break through the shell but failed.

FIG. 3.—20-day-old Rhode Island Red embryo with grafted White Leghorn embryonic forebrain region. Head quite normal, except for some retardation in development. The entire head including the mandible has developed from the graft. Head rotated nearly 90° in relation to a second mandible which probably originated from the host. Note a deep groove in the region of healing.

The healing process at the cut surfaces brought in apposition takes place almost immediately in autografts where apposition is more easily attained. In most instances, the line of fusion between the graft and the host tissues forms no barrier to the ingrowing blood vessels. In a few cases, there is a lag in the establishment of vascularity in the graft as compared with the host. Extravasation may occur, blood filling the forebrain vesicles; however, this is resorbed in about a week.

The results obtained depend, in the main, on the precision secured at each step of the operation. Perfect healing is obtained less frequently in homografts, where it is more difficult to secure perfect apposition of the cut surfaces of the graft and

the host tissues. The greatest trouble stems from the fact that the cut surfaces of the nerve tissues at both ends close in very quickly and heal separately, thus completely occluding the neural canal, with resultant hydrocephalus. Further growth and development of the grafted forebrain region, however, may be perfectly regular in every respect, and when the grafted embryos survive to the last day of incubation, some attempt unsuccessfully to break the shell.

Homografts were made with the same breed as the host and with different breeds. Figures 1–3 show three animals in which the host tissues are of different breeds, and indicate the degree of success so far attained. When one considers the probabilities for damage inherent to this type of work, when handling tissues of near-fluid constituency, it is remarkable that comparatively normal development ensues.

Deformities, attributable to faulty technique, were a common occurrence and affected the brain (hydrocephalus, failure of the cranial bones to cover the brain mass, etc.), the eyes (defective and underdeveloped eyeball), and the mouth parts. If the host belonged to a colored breed and the graft to a noncolored one (Barred Rock Black and White Leghorn), the chromatophores invaded the graft, so that the feathers in the head region were of the same color as those of the host animal.

The transplantation of the forebrain region can result in normal development and differentiation of the graft if the transplantation is completed before the establishment of vascularity in the host. There seems to be no difference in the degree of success of the operation whether the grafted tissue belong to the same embryo or to one belonging to a different breed of chicken.

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